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<p>(21) International Application Number: PCT/EP98/03791</p> <p>(22) International Filing Date: 22 June 1998 (22.06.98)</p> <p>(30) Priority Data: 97870092.0 20 June 1997 (20.06.97) EP (34) Countries for which the regional or international application was filed: BE et al.</p> <p>(71) Applicant (for all designated States except US): INNOGENET-ICS N.V. [BE/BE]; Industriepark Zwijnaarde 7, P.O. Box 4, B-9052 Ghent (BE).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): LORRE, Katrien [BE/BE]; Lieven De Winnestraat 59, B-9000 Gent (BE). SABLON, Erwin [BE/BE]; Robbroeklaan 1A, B-1785 Merchtem (BE). BUYSE, Marie-Ange [BE/BE]; Burg. E. Ronsestraat 23, B-9820 Merelbeke (BE). BOSMAN, Alfons [BE/BE]; Hulst 165, B-1745 Opwijk (BE).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: B7-BINDING MOLECULES FOR TREATING IMMUNE DISEASES</p> <p>(57) Abstract</p> <p>The present invention relates to molecules, such as diabodies, tri- and tetravalent antibodies and small antigen binding peptides, which can cross-link or cross-react with the costimulatory molecules B7.1 and B7.2 expressed on professional antigen presenting cells (APCs) leading to the inhibition of antigen-specific T cell activation. The present invention also concerns methods to produce these molecules and use of these molecules to treat diseases, such as transplant rejection, graft versus host disease (GVHD), allergy and autoimmune diseases, where antigen-specific T cell activation is pathogenic.</p> <p><i>1 p5, tetravalent</i></p>		

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B7-BINDING MOLECULES FOR TREATING IMMUNE DISEASESFIELD OF THE INVENTION

5           The present invention relates to molecules which can cross-link or cross-react with the costimulatory molecules B7.1 and B7.2 expressed on professional antigen presenting cells (APC's) leading to the inhibition of antigen-specific T cell activation. The present invention also concerns methods to produce these molecules and use of these molecules to treat diseases, such as transplant rejection, graft versus host  
10       disease (GVHD), allergy and autoimmune diseases, where antigen-specific T cell activation is pathogenic.

BACKGROUND OF THE INVENTION

15       \* Molecules which cross-link or cross-react with B7.1 and B7.2

          It is known that the use of monoclonal antibodies (mAb's) for diagnostic or therapeutic purposes *in vivo* is limited because of their nature (the majority are murine mAb's), their suboptimal stability and affinity and their large molecular size.  
20       In order to solve these problems several modified antibodies, antibody constructs and peptide and nonpeptide antigen binding fragments have been developed by bioengineering or chemical methods. Murine mAb's were made less antigenic to humans by CDR grafting (Winter and Harris, 1993). mAb's were made more effective by conjugating chemotherapeutic drugs and other toxins to the antibodies (Ghetie and  
25       Vitetta, 1994). Another method to produce more effective antibodies is the development of bispecific antibody constructs capable of simultaneously binding two different epitopes on the same- or different antigens. These bispecific antibodies have been produced using a variety of methods: a) antibodies of different specificities or univalent fragments of pepsin-treated antibodies of different specificities have been  
30       chemically linked (Fanger et al., 1992); b) two hybridomas secreting antibodies of different specificity have been fused and the resulting bispecific antibodies from the

mixture of antibodies were subsequently isolated; c) genetically engineered single chain antibodies have been used to produce non-covalently linked bispecific antibodies (e.g. diabodies (Holliger et al., 1993), minibodies (Kostelny et al., 1992; Pack et al., 1993) and tetravalent antibodies (Pack et al; 1995; WO 96/13583 to Pack) or covalently-linked bispecific antibodies (e.g. chelating recombinant antibodies (Kranz et al., 1995), single chain antibodies fused to protein A or Streptavidin (Ito and Kurosawa, 1993; Kipriyanov et al., 1996) and bispecific tetravalent antibodies (EP 0517024 to Bosslet and Deeman; Morrison, 1993). Recently, also trivalent antibody constructs, named triabodies, have been described (Kortt et al., 1997; Iliades et al., 1997). These trivalent constructs may have a higher avidity in comparison to bivalent constructs and may be useful for diagnostic or therapeutic purposes *in vivo*. Phage display of Ab combinatorial libraries resulting in the production of high-affinity antibodies and screening of random DNA sequence phage display libraries for small antigen-binding peptides that mimic the antigen specific binding activity of an antibody are other approaches to produce more effective antigen-binding molecules (for review see Hayden et al., 1997 and Hoogenboom, 1997). Also the production of low-molecular-weight nonpeptide molecules, which mimic the chemical structure and biological activity of their peptide counterpart, but have longer biological half-lives and better oral bioavailability, is an attractive alternative to obtain effective antigen-binding molecules (Wiley and Rich, 1993; Wendolowski et al., 1993 and Lybrand, 1995). Peptidic or non-peptidic molecules which are derived from 'High Throughput Screening' (HTS) of chemical or natural libraries and inhibit protein-protein interactions (Sarubbi et al., 1996) can also be used as effective antigen-binding molecules. Also the generation of hybridomas, derived from immunized transgenic mice, containing large sections of the human immunoglobulin (Ig) gene loci in the germ line, integrated by the yeast artificial chromosomal (YAC) technology, is a method to obtain effective blocking antibodies (Mendez et al, 1997). Although antibodies against B7 antigens have been described (as for example the macaque antibodies to human B7. and B7.2 described in WO96/40878 to Anderson et al.), no prior art exists regarding the production and existence of molecules which cross-link or cross-react with B7.1 and B7.2 and which do not comprise a variable domain of a

monkey antibody or the extracellular domain of the B7-binding molecules CTLA4 or CD28.

\* T cell activation

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Activation of T cells is the result of ligand-receptor interactions. The TcR/CD3 complex has two functions in antigen-induced activation: a recognition function in which a specific antigen is recognized in the context of the appropriate MHC molecule, and a signaling function in which the recognition event is transmitted across the plasma membrane (Imboden et al., 1985; Weiss and Imboden, 1987). However, T cells require a second signal to induce proliferation and maturation into effector cells. This costimulatory signal is provided by the cell surface of APC's (Springer et al., 1987). Intercellular signaling after TcR/MHC-peptide interaction in the absence of the costimulatory signal results in T cell inactivation in the form of clonal anergy (Mueller et al., 1989). The interaction of a number of accessory molecules present on the cell surface of T cells with known ligands on the APC have been implicated in providing the costimulatory signal in T cell activation: CD2 with its ligand CD58 (LFA-3), CD11a/CD18 (LFA-1) with CD54 (ICAM-1), CD28 with B7, and CD29/CD49d 5VLA-4 with VCAM-1 (Selvaraj et al., 1987; Springer, 1990; Marlin and Springer, 1987; Van Noesel et al., 1988; Linsley et al., 1990; Damle et al., 1991).

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To date, the best candidate costimulatory signal leading to full T cell activation, or to T cell anergy, is generated by interaction of CD28 on the T cells with the B7 costimulatory molecules on APC's. *In vitro* studies have demonstrated that signaling via the CD28 costimulatory pathway can prevent the induction of anergy. Harding and coworkers (1992) have demonstrated that activation of mouse T cell clones with anti-CD3 Mab in the absence of APC results in anergy. However, under these conditions cross-linking of the CD28 molecule using anti-CD28 Mab could prevent anergy induction. In contrast, when the same T cell clones were stimulated with anti-CD3 Mab and competent APC, addition of Fab fragments of anti-CD28 Mabs caused T cell anergy. In addition, it has been demonstrated that mouse fibroblasts co-transfected with MHC-DR-7 and human B7.1, but not with ICAM-1, could prevent anergy induction

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of DR-7-specific alloreactive human T cells (Boussiotis et al., 1993a).

\* The B7 costimulatory molecules

5       To date, two members of the B7 family have been molecularly cloned and functionally characterized: B7.1 (CD80), originally named B7/BB1, and B7.2 (CD86), originally named B70.

10       B7.1 is a monomeric transmembrane glycoprotein with an apparent molecular mass of 45-65 kDa and is a member of the immunoglobulin superfamily (Freeman et al., 1989). It was initially reported that the expression of the B7.1 molecule was restricted to activated B cells (Freeman et al., 1989) and monocytes stimulated with IFN- $\gamma$  (Freedman et al., 1991). More recently, B7.1 expression has also been found on cultured peripheral blood dendritic cells (Young et al. 1992) and on *in vitro* activated T cells (Azuma et al., 1993a). The expression of the B7.1 molecule in a number of  
15       normal and pathological tissues has been examined by immunohistochemistry using the anti-B7.1 Mab B7-24 (Vandenberghe et al., 1993). In addition to the staining of activated B cells, the B7.1 molecule was shown to be constitutively expressed *in vivo* on dendritic cells in both lymphoid and non-lymphoid tissue. Monocytes/macrophages were only found to be positive under inflammatory conditions and endothelial cells  
20       were always negative. Interestingly, the number of B7.1 positive cells in skin lesions of patients with acute GVHD was strongly increased compared to normal skin.

25       B7.2 is also a transmembrane glycoprotein with an apparent molecular mass of approximately 70 kDa and is also a member of the immunoglobulin superfamily (Freeman et al., 1993; Azuma et al., 1993b). The B7.2 molecule seems to have a very similar histo-distribution pattern to B7.1, with the exception that the induction of cell-surface expression seems to be faster (Freeman et al., 1993) and that it is present on freshly isolated monocytes (Azuma et al., 1993b). Also the expression of the B7.2 molecule was found to overlap to a large extent with B7.1, with minor differences in some B cell subsets in and around germinal centers, which most likely reflects  
30       different activation status of the B cells (de Boer, unpublished results).

In addition to the two well-characterized B7 molecules at least two other B7-like

molecules have been described. One shares structural homology with B7.1 since it is recognized by the BB1 anti-Mab. This molecule has been identified on activated B cells and activated keratinocytes (Boussiotis et al., 1993b). This molecule can bind to CD28 but not CTLA-4 (see below). However, this molecule does not seem to be able to stimulate T cells via CD28. A second B7-like molecule has been described on EBV-transformed B cell lines and functionally interacts with CTLA-4 on T cells, resulting in apoptosis of previously activated T cells (Gribben et al., 1995).

\* CD28 and CTLA-4: B7-binding molecules.

CD28, a homodimeric transmembrane glycoprotein with an apparent molecular mass of 44 kDa and a member of the immunoglobulin superfamily, is expressed on approximately 95% of the CD4-positive T cells and 50% of the CD8-positive T cells (June et al., 1990). CD28 regulates a signal transduction pathway distinct from that induced by the TcR/CD3 complex (Vandenberghe et al., 1992). Many studies indicate that costimulation of T cells by cross-linking the CD28 molecule with Mab results in a greatly enhanced activation, which is accompanied by the production of large amounts of interleukin-2 (IL-2) (Thompson et al., 1989; June et al., 1989) and other cytokines. Furthermore, anti-CD28 Mabs can be replaced by B7, the natural ligand for CD28 (Linsley et al., 1991; Gimmi et al., 1991; de Boer et al., 1992). The B7-CD28 interaction can result in a strong proliferative (de Boer et al., 1992) as well as cytolytic T cell response (Van Gool et al., 1993). Ligation of CD28 can generate two signal transduction pathways: a calcium-independent signal which is probably the most important and a calcium-dependent signal. Cyclosporin A (CsA), which is currently the most important immunosuppressive drug, can only completely prevent T cell activation when this activation is totally dependent upon a calcium calcineurium-mediated intracellular event (Bierer et al., 1993). Consequently, the activation of human T cells through the TcR in the presence of costimulation via B7-CD28 is resistant to inhibition by CsA (June et al., 1987).

The CTLA-4 molecule is closely related to CD28. The amino acid sequences of both CTLA-4 and CD28, predicted from their corresponding genes which are located

on the same chromosome, show a high degree of homology, especially in the transmembrane domain and some defined regions of the extracellular domain (Harper et al., 1991). Furthermore, both CTLA-4 and CD28 bind the B7 molecules (Linsley et al., 1991; Freeman et al., 1993; Azuma et al., 1993b). However, CTLA-4 has, compared to CD28, a 10 to 20-fold higher affinity for B7 (Linsley et al., 1991), and, whereas CD28 is constitutively expressed at relatively high levels on T cells, CTLA-4 is only expressed at low levels on activated T cells (Linsley et al., 1992). mRNA for CTLA-4 can be detected shortly after activation of T cells, cell surface expression is only found after 2-4 days in culture (Linsley et al., 1992). The exact function of CTLA-4 has been, until recently, controversial (summarized in Science Perspective, Allison and Krummel, 1995 and Refs therein). Antibodies to CTLA-4, when co-administered with suboptimal concentrations of anti-CD28 Mab, enhance T cell proliferation. However, when cross-linked, antibodies to CTLA-4 profoundly inhibit the proliferation of naive T cells. Moreover, CTLA-4 cannot replace CD28 to provide the costimulatory signal in CD28-deficient mice. Taken together, these data suggest that blockade of CTLA-4 binding to its ligand(s) removes inhibitory signals, whereas aggregation (i.e. cross-linking) of CTLA-4 induces inhibitory signals which down-regulate T cell responses. This hypothesis was confirmed by the phenotype of CTLA-4 deficient mice which exhibit a lymphadenopathy of extreme magnitude (Waterhouse et al., 1995). The peripheral organs of these mice contain 5-10 times the normal number of lymphocytes, the vast majority of which are activated T cells as indicated by the expression of various activation markers. The latter findings clearly support the hypothesis that the function of CTLA-4 is to switch-off T cell responses which has, as we will discuss below, important implications for the design of optimal immunosuppressive strategies based on blocking the B7-CD28 interaction.

#### \* Transplant rejection

Recognition of alloantigen on transplanted tissue by CD4<sup>+</sup> helper T cells leads to the induction of a variety of lymphokines. These lymphokines subsequently drive the maturation of precursor to effector cells of different immune functions. Without



immune suppression this cascade of events will result in rejection of the grafted tissue. The current strategies for prevention of graft rejection are based on the use of broad acting immunosuppressive drugs such as cyclosporin and corticosteroids. These drugs not only suppress the immune reaction to the alloantigen on the transplanted tissue, but also suppress the immune response to bacterial or viral pathogens. In addition, in order for the graft to survive, these drugs must often be taken over long periods of time and therefore increase the risk of serious infections, nephrotoxicity and cancer. Thus, the immunological system of the patient after organ transplantation is placed in a dangerously unstable balance: a low immunosuppression results in transplant rejection, whereas a high immunosuppression increases the risk of fatal microbial infections. Due to a better understanding of the mechanism of action of the currently used immunosuppressive drugs and the fine-tuning of dosing regimes, acute rejection is not a major problem anymore. The success rate (one year survival) of for instance kidney allografts is more than 95%. However, despite the progress in the management of acute rejection, allograft loss after a few years due to what is called chronic vascular rejection remains a large problem, for which no effective treatments are available. Consequently, it has become crucial to develop and evaluate alternative approaches. Since donor-antigen-specific lymphocyte hyporeactivity has been demonstrated in patients without chronic rejection of a transplanted lung, in contrast to those in whom chronic rejection did develop (Reinsmoen et al., 1994), the optimal therapy to prevent graft rejection should be based on the induction of specific T cell tolerance to the donor tissue. Thus an ideal drug treatment should induce clonal un-responsiveness of donor-reactive T cells (anergy), without the need for long-term, non-specific immunosuppression and without the occurrence of chronic vascular rejection.

Furthermore, several *in vivo* models have shown that induction of prolonged graft acceptance is possible by interruption of the B7.1/B7.2-CD28 pathway. Treatment with CTLA-4Ig (50 µg/d) given every other day for 14 days immediately after xenogeneic human pancreatic islet transplantation in mice resulted in long-term graft survival (Lenschow et al., 1992). However, 90% of fully mismatched rat cardiac allografts were rejected in rats treated intraperitoneally (IP) with 500 µg/d CTLA-4Ig during 7 days

(Turka et al., 1992). CTLA-4Ig (50 µg/d), given intravenously (IV) at time of transplantation and then IP every other day on days 2 through 12, prolonged cardiac allograft survival in mice, but failed to prolong the survival of primary skin grafts (Pearson et al., 1994). The discrepancies between rat and mouse cardiac transplantation models can be explained by differences in immunogenicity of allografts, the differential impact of costimulatory adhesion molecules other than B7.1/B7.2, altered pharmacokinetics, and/or changed immunogenicity and affinity of the CTLA-4Ig administered. Blockage of the CD28-pathway with CTLA-4Ig (500 µg/d IV or IP on days 0,1,2,3,4,6, and 8) resulted in significant prolongation of small bowel transplant survival in rats compared to controls, although all grafts were rejected after 15 days (Pescovitz et al., 1994). Finally, treatment with CTLA-4 Ig (100 or 250 µg/d IP for 4 weeks) could reduce lethal murine GVHD in recipients of fully allogeneic bone marrow and significantly prolonged survival rates with up to 63% of mice surviving greater than 3 months post-transplantation (Blazar et al., 1994). The failure of CTLA-4Ig alone to induce anergy *in vitro* and *in vivo*, can most likely be explained by persistent IL-2 production, induced by TCR triggering in combination with signaling from other accessory molecules on APC.

Taken together, an ideal drug treatment to prevent transplant rejection should induce donor-specific T cell anergy and reduce the need for long term non-specific immunosuppression. In this regard, no prior art exists demonstrating that administration of a molecule which cross-links, or cross-reacts with, B7.1 and B7.2 and which does not comprise the extracellular domain of CTLA4 or CD28, can, possibly in combination with a reduced amount of immunosuppressive agents, prevent allograft rejection.

#### \* Graft-versus-host disease:

Graft-versus-host disease (GVHD), which is a major complication during allogeneic bone marrow transplantation, is initiated by immunocompetent donor T cells which recognize alloantigens of an immunocompromised host. GVHD can be classified into acute- and chronic GVHD on the basis of histologic characteristics.

Acute GVHD involves necrosis of the epithelium of the skin, liver and gastrointestinal tract and is clinically characterized by skin rash, jaundice and diarrhea. Chronic GVHD involves fibrosis and atrophy of the same organs as for acute GVHD and may result in complete dysfunction of these affected organs. Treatment with both CsA and methotrexate have been shown to reduce the risk of acute GVHD. Treatment with prednisone, alone or in combination with azathioprine, has been shown to effectively reverse chronic GVHD in 50-75% of patients. Furthermore, incubating the donor marrow in vitro with anti-T-cell mAb's plus complement or anti-T-cell immunotoxins has successfully depleted the marrow of T cells and lowered the incidence of GVHD. However, patients receiving T cell-depleted marrow did have an increased risk of rejection, higher relapse rate of leukemia and increased risk of fungal infection which indicated that donor T cells also play a protective role. Furthermore, neither immunosuppressive therapy nor T cell depletion specifically prevent the recognition of host alloantigens by donor T cells. An approach which would specifically target this recognition would preserve the protective donor T cells and eliminate the pathogenic T cells. Recently, Gribben et al. (1996) demonstrated that host alloantigen-specific anergy in human donor T cells could be induced *ex vivo* by using anti-B7.1 plus anti-B7.2 mAb's or a CTLA4-Ig fusion protein. However, no prior art exists demonstrating that administration of a molecule which cross-links, or cross-reacts with, B7.1 and B7.2 and which does not comprise the extracellular domain of CTLA4 or CD28, can, possibly in combination with a reduced amount of immunosuppressive agents, more efficiently prevent the alloantigen-specific activation of donor T cells and thus GVHD.

#### \* Autoimmune diseases

A number of studies indicate that costimulation through CD28 ligation might be the initiating event in autoimmunity. The potential of both a primary signal via the TcR and B7.1 as a costimulatory signal for the generation of autoimmune diabetes has clearly been proven with transgenic mice (Guerder et al., 1994; Harlan et al., 1994). In these studies, it is hypothesized that tolerance to peripheral antigens is induced by triggering the TcR in the absence of essential costimulatory signals. Mice expressing

both B7.1 and a high level of primary antigens (MHC molecules or viral glycoproteins) on pancreatic beta cells developed autoimmune diabetes. The critical role of the absence of B7.1-mediated costimulation in the induction and maintenance of tolerance to peripheral antigens, and of the B7.1-mediated signaling in the breakdown of T cell non-responsiveness, causing autoimmunity, was obvious.

The role of the B7.1/B7.2-CD28 interaction in the chronic activation state of T cells, which have been implicated in autoimmune diseases, has been strongly suggested in various studies. Using immunohistochemical techniques, strong B7.1 expression has been found in lesions of autoimmune diseases, such as rheumatoid arthritis and psoriasis. Furthermore, it has been demonstrated that blocking B7.1/B7.2-CD28 interaction could block auto-antibody production and prolongation of life in a murine model of autoimmune disease that closely resembles systemic lupus erythematosus in humans (Finck et al., 1994). However, no prior art exists demonstrating that administration of a molecule which cross-links, or cross-reacts with, B7.1 and B7.2 and which does not comprise the extracellular domain of CTLA4 or CD28; can, possibly in combination with a reduced amount of immunosuppressive agents, more efficiently prevent autoimmune disorders.

#### \* Allergy

Initial expression to allergen in atopic individuals results in IgE production by B cells. This IgE will first sensitize local mast cells, and 'spill-over' IgE enters the circulation and binds on receptors on circulating basophils and tissue mast cells throughout the body. The IgE production is controlled by T helper 2 (Th2) cells, as IL-4 promotes the IgE synthesis by B cells.

The role of the B7.1/B7.2-CD28 interaction in the development of an allergic reaction has been strongly suggested in various studies. It was shown by Keane et al (1997) that the B7-CD28/CTLA-4 costimulatory pathway is required for the development of Th2 mediated allergic airway responses to inhaled antigens. In this study, sensitized A/J mice develop significant increases in airway responsiveness, bronchioalveolar lavage eosinophils, serum IgE levels and Th2-associated cytokine production following

aspiration challenge with OVA. Administration of CTLA-4 Ig in this murine model, either before Ag sensitization or before pulmonary Ag challenge, abolished antigen induced hyperresponsiveness and pulmonary eosinophils. The level of Th2 cytokine, IL-4 and of antigen specific Ab isotypes IgG1 and IgE was significantly decreased. Furthermore, Krinzman et al. (1996) showed that blockade of costimulation with CTLA-4-Ig inhibits airway hyperresponsiveness, inflammatory infiltration, expansion of thoracic lymphocytes, and allergen-specific responsiveness of thoracic T cells in a murine model of allergic asthma. However, no prior art exists demonstrating that administration of a molecule which cross-links, or cross-reacts with, B7.1 and B7.2 and which does not comprise the extracellular domain of CTLA-4 or CD28, can, possibly in combination with a reduced amount of immunosuppressive agents, more efficiently prevent allergic reactions.

#### AIMS OF THE INVENTION

It is clear from the literature that blocking B7.1 alone results in partial inhibition of T cell activation. Indeed, activation of T cells by alloantigen-expressing monocytes appears predominantly dependent on B7.2 costimulation. Furthermore, we have previously demonstrated that, during a mixed lymphocyte culture (MLC) with monocytes as stimulator cells, anti-B7.2 mAb alone could strongly but not completely inhibit the proliferative response. Only CTLA4-Ig, which binds both B7.1 and B7.2 (i.e. cross-reacts with B7.1 and B7.2) or a combination of anti-B7.1 plus anti-B7.2 mAb's gave maximal inhibition.

Moreover, and as already indicated above, several recent *in vivo* models have shown that CTLA4-Ig alone fails to induce anergy. The latter finding can most likely be explained by a persistent IL-2 production, induced by TCR triggering in combination with signalling from other accessory molecules such as LFA-1 (CD11a/CD18), LFA-3 (CD58), ICAM's (CD54, CD102, CD50) or others on APC's. Indeed, signalling through the IL-2 receptor gamma chain shortly after TCR triggering is able to prevent induction of anergy (Boussiotis et al., 1994; Van Gool et al., 1994). This phenomenon was illustrated in an experiment in which purified T cells were cultured for six days with

alloantigen-expressing EBV-transformed B cells. After 6 days in the presence or absence of blocking agents, cells were harvested, cultured for 2 days in plain medium without any additions and restimulated with the same EBV cell line. Addition of CTLA4-Ig or the combination of anti-B7.1 plus anti-B7.2 did induce hyporesponsiveness. However, only the addition of CsA to the B7.1/B7.2 mAb's resulted in alloantigen-specific anergy. In another experiment, the combination of anti-B7.1 plus anti-B7.2 and CsA, but not each of them separately, prevented subsequent CTL generation in a secondary MLR performed with the same EBV B cell line but in the absence of the blocking agents. These experiments clearly demonstrate the superiority of the combined B7.1/B7.2 antibodies versus CTLA4-Ig. Therefore, the present invention aims at providing a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise the extracellular domain of CTLA-4 or CD28.

There are several other important reasons why a molecule which can cross-link, or cross-react with, B7.1 and B7.2, and which does not comprise the extracellular domain of CTLA-4 or CD28 is a preferred therapeutic to block accessory molecule function:

1) Such a molecule can be produced in a bacterial expression system. This ensures low production and quality control costs compared to CTLA4-Ig that requires mammalian fermentation.

2) An optimal blocking agent for B7.1 and B7.2 should have a high affinity for both B7 molecules. CTLA4-Ig is a high affinity ligand for both B7 molecules, however this high affinity is due to a very high on-rate. CTLA4-Ig has also an extremely fast off-rate (Linsley et al., 1995). Since both B7 molecules are not modulated from the cell surface after ligation with CTLA4-Ig or with specific mAb's, a molecule with a very slow off-rate is the ideal blocking agent for application *in vivo*. In this respect, molecules which can cross-link, or cross-react with, B7.1 and B7.2, and which do not comprise the extracellular domain of CTLA-4 or CD28 prove to be good therapeutic candidate molecules, because we have means to improve the binding characteristics of mAb's via the mutagenesis of the CDR regions. In contrast, improving the binding characteristics of CTLA4-Ig is extremely difficult given the nature of the molecule.

3) There are at least 3 CTLA4-Ig counter receptors. If any of these CTLA4-Ig counter

receptors can interact with CTLA-4 but not with CD28, then the usage of CTLA4-Ig may mediate deleterious effects *in vivo*. Because CTLA4 on activated T cells functions as a terminator of T cell activation one would prefer not to block a ligand that can interact with CTLA4 but not with CD28. Such a CTLA4 ligand was demonstrated recently (Gribben et al., 1995). This as yet uncharacterised CTLA4 ligand induced antigen-specific apoptosis of previously activated T cells. Neither B7.1 nor B7.2 mediate apoptosis via CTLA4. Activated T cells were rechallenged with an alloantigen-bearing EBV-positive B cell line in the presence of blocking agents. The simultaneous addition of mAb's to B7.1 and B7.2, but not each of them separately, strongly inhibited T cell proliferation and IL-2 production and, most interestingly, strongly induced apoptosis of more than 90% of the cells. Therefore, the present invention aims at providing a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise the extracellular domain of CTLA-4 or CD28.

Primatized antibodies derived from macaque monoclonal antibodies which bind B7.1, and possibly B7.2, are described in WO 96/40878 to Anderson et al. However, because of the complexity and ethical constraints of working with monkeys, the present invention aims at providing, in a less-complex, more-ethically acceptable and more elegant manner, a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody. In this regard, Zhang and Johnson (1997) have also shown that macaque B7.1 and B7.2 molecules are highly homologues (an overall amino acid homology of greater than 90%) to their human counterparts and are specifically recognized by neutralizing murine anti-human B7.1 and B7.2 monoclonal antibodies. These findings strongly suggest that macaques will develop an auto-immune response upon injecting/immunizing them with human B7.1 and B7.2 as described in WO 96/40878 to Anderson et al. which will result in immunosuppression. In other words, the anti-human B7.1 and B7.2 macaque antibodies will bind to the monkey's own B7.1 and B7.2 molecules which will result in the neutralization of the monkey's B7-mediated T cell activation. The present invention aims at overcoming the latter complex and ethical constraints.

More particularly, the present invention aims at providing a molecule which

cross-links, or cross-reacts with, B7.1 and B7.2, as described above and which comprises at least one first domain which binds B7.1 or B7.2 or cross-reacts with B7.1 and B7.2, at least one second domain which binds B7.1 or B7.2 or cross-reacts with B7.1 and B7.2, and optionally a third domain which couples the first and the second domain(s).

More specifically, the present invention aims at providing a molecule as described above wherein said first and second domain is a low-molecular weight nonpeptide molecule or a polypeptide such as an antibody or a humanized antibody, a single chain fragment or another fragment thereof which has largely retained the specificity of said antibody, or a small antigen-binding peptide which is neither an antibody nor derived from an antibody, and wherein said third domain is a polypeptide, any chemical coupling agent or any oligomerization domain.

More particularly, the present invention aims at providing bispecific antibodies such as miniantibodies, diabodies and bispecific tetravalent antibodies, trivalent antibodies, bispecific small antigen-binding peptides and bispecific low molecular weight nonpeptide molecules.

Furthermore, the invention aims at providing a method to produce said molecule which cross-links, or cross-reacts with, B7.1 and B7.2, as described above.

The invention also aims at providing a composition comprising said molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise the extracellular domain of CTLA-4 or CD28, or a variable domain of a monkey antibody, in a pharmaceutically acceptable excipient which can be used as a medicament.

The invention also aims at providing an alternative and better method to inhibit antigen-specific T cell activation and/or to treat diseases of the immune system such as allograft rejection, GVHD, allergy and autoimmune diseases by using a molecule which cross-links, or cross-reacts with, B7.1 and B7.2 as described above. Moreover, the present inventors have been able to prove that, surprisingly, cross-linking of B7.1 and B7.2 using a molecule as described above leads to the inhibition of immunoactivators such as IL-12. This characteristic is a valuable asset in order to suppress T cell mediated immune responses.



Furthermore, and as we indicated above, in one of our experiments, the combination of anti-B7.1 plus anti-B7.2 and CsA, but not each of them separately, prevented subsequent CTL generation in a secondary MLR performed with an EBV-transformed cell line in the absence of the blocking agents. Most importantly, even when the addition of the antibodies and CsA was delayed for 24 hours, the generation of CTL activity after restimulation was almost completely blocked. Taken together, these experiments clearly demonstrate, in addition to prevent costimulation via B7.1/2 signalling, the need to interfere with the TCR signal using CsA. Therefore, the present invention aims at providing a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise the extracellular domain of CTLA-4 or CD28 or a variable domain of a monkey antibody which possibly can be administered in conjunction with CsA or other immunosuppressive chemicals in order to inhibit antigen-specific T cell activation and/or to treat diseases of the immune system such as allograft rejection, GVHD, allergy and autoimmune diseases.

All the aims of the present invention are considered to have been met by the embodiments as set out below.

#### BRIEF DESCRIPTION OF FIGURES

20

Figure1 shows the Map of baculo transfer vector pVL-Fc

The vector contains :

- \* a E.coli origin of replication and an ampiciline resistance prokaryotic expression unit for propagation of the plasmid DNA in E.coli.
- \* baculoviral expression cassette : baculoviral polydrin promoter (Ppolh) followed by a BamHI cloning site allowing upstream fusion of a cDNA encoded ORF (e.g. hB7-ED) to a cDNA sequence encoding the Fc region (Hinge-CH2-CH3) of a human IgG $\gamma$ 1.
- \* baculoviral polyhedrin locus genome sequences flanking the baculovirus expression cassette

30

Figure 2 shows the protein sequence of the hB7.1Fc soluble fusion protein

Amino Acids 1-34 : potential eukaryotic secretory signal peptide

Amino Acids 35-241 : extracellular domain of hB7.1 protein (mature protein)

Amino Acids 242-248 : introduced by PCR cloning strategy

5 Amino Acids 249-480 : human IgG1-Fc (Hinge-CH2-CH3)

Figure 3 shows the protein sequence of the hB7.2Fc soluble fusion protein

Amino Acids 1-16 : potential eukaryotic secretory signal sequence (Azuma et al., Nature, 1993)

10 Amino Acids 17-239 : extracellular domain of hB7.2 protein (mature protein)

Amino Acids 240-245 : introduced by PCR cloning strategy

Amino Acids 246-477 : human IgG1-Fc (Hinge-CH2-CH3)

Figure 4 shows the protein sequence of the hB7.1glu-glu soluble fusion protein

15 Amino Acids 1-34 : potential eukaryotic secretory signal peptide

Amino Acids 35-242 : extracellular domain of hB7.1 protein (mature protein)

Amino Acids 243-251 : glu-glu detection/purification tag

Figure 5 shows the protein sequence of the hB7.2his soluble fusion protein

20 Amino Acids 1-23 : potential eukaryotic secretory signal peptide (Azuma et al., Nature, 1993)

Amino Acids 24-238 : extracellular domain of hB7.2 protein (mature protein)

Amino Acids 239-244 : histidine detection/purification tag

25 Figure 6 shows gel filtration profile of sB7.1 gluglu (b) and MW markers (a) 1 = 670 kD, 2 = 158 kD, 3 = 44 kD, 4 = 17 kD, 5 = 1.3 kD

Figure 7 shows gel filtration profile of sB7.2 His6 (b) and MW markers (a) 1 = 670 kD, 2 = 158 kD, 3 = 44 kD, 4 = 17 kD, 5 = 1.3 kD

30

Figure 8 shows the neutralizing activity of scFv B7-24 compared to parent B7-24 mAb

Figure 9 shows the PhagemidpCES1 :

antibody genes :  $V_L-C_L$ , variable (V) and constant (C) region of the light chain;  $V_H-C_{H1}$ , variable and first constant region of the heavy chain; *PlacZ*, promoter; rbs, ribosome binding site; S, signal sequence; H6, six histidines stretch for IMAC purification; tag, c-myc-derived tag; amber, amber codon that allows production of soluble Fab fragments in non-suppressor strains, gIII, gene encoding one of the minor coat proteins of filamentous phage. Restriction sites used for cloning are indicated.

Figure 10 shows sequences of hybrid immunoglobulin light chain/CTLA-4 molecule

Figure 11 shows the experimental strategy for 'random DNA shuffling'

Figure 12 shows sequences of the CTLA-4CDR3/ $V_k$  light chain spiking oligonucleotides

Figure 13 shows sequences of CTLA-4 CDR3/ $V_\lambda$  light chain spiking oligonucleotides

Figure 14 shows a BiTAAb molecule

Figure 15 shows the DNA sequence of the BiTAAbB7-24-1G10H6 molecule

Nucleotides 1-72 : pelB signal sequence

Nucleotides 73- 415 : VH region anti B7.1 Mab (B7-24)

Nucleotides 416 - 460 :  $(G_4S)_3$  flexible linker

Nucleotides 461 -787 : VL region anti B7.1 Mab (B7-24)

Nucleotides 788 - 820 : Human IgG3 hinge region

Nucleotides 821 - 925 : Helix-Turn-Helix Dimerisation Domain

Nucleotides 926 - 958 : Human IgG3 hinge domain

Nucleotides 959 - 1325 : VH region anti B7.2 Mab (1G10)

Nucleotides 1326 - 1369 :  $(G_4S)_3$  flexible linker

Nucleotides 1370- 1708 : VL region anti B7.2 Mab (1G10)

Nucleotides 1709 - 1726 : His6 Tag

Figure 16 shows the Protein Sequence of the BiTabB7-24-1G10H6 molecule

Amino Acids 1-24: pelB signal sequence

Amino Acids 25- 138: VH region anti B7.1 Mab

Amino Acids 139- 153: (G<sub>4</sub>S)<sub>3</sub> flexible linker

5 Amino Acids 154 - 262: VL region anti B7.1 Mab

Amino Acids 263- 273: Human IgG3 hinge region

Amino Acids 274-308: Helix-Turn-Helix Dimerisation Domain

Amino Acids 309-319: Human IgG3 hinge domain

Amino Acids 320-446 : VH region anti B7.2 Mab

10 Amino Acids 447- 461: (G<sub>4</sub>S)<sub>3</sub> flexible linker

Amino Acids 462- 574: VL region anti B7.2 Mab

Amino Acids 575- 580: His6 Tag

Figure 17 shows DNA sequence the BiTab1G10-B7-24H6 molecule

15 Nucleotides 1 - 360 : VH region anti B7.2 Mab (1G10)

Nucleotides 361 - 405 : (G<sub>4</sub>S)<sub>3</sub> flexible linker

Nucleotides 406 - 744 : VL region anti B7.2 Mab (1G10)

Nucleotides 745 - 777 : Human IgG3 hinge region

Nucleotides 778 - 882 : Helix-Turn-Helix Dimerisation Domain

20 Nucleotides 883 - 915 : Human IgG3 hinge domain

Nucleotides 916 - 1278 : VH region anti B7.1 Mab (B7-24)

Nucleotides 1279 - 1314 : (G<sub>4</sub>S)<sub>3</sub> flexible linker

Nucleotides 1314 - 1650 : VL region anti B7.1 Mab (B7-24)

Nucleotides 1651 - 1670 : His6 Tag

25

Figure 18 shows Protein Sequence of the BiTab1G10-B7-24H6 molecule

Amino Acids 1 - 120 : VH region anti B7.2 Mab

Amino Acids 120 - 135 : (G<sub>4</sub>S)<sub>3</sub> flexible linker

Amino Acids 136 - 248 : VL region anti B7.2 Mab

30 Amino Acids 249 - 259 : Human IgG3 hinge region

Amino Acids 260 - 285 : Helix-Turn-Helix Dimerisation Domain

Amino Acids 286 - 305 : Human IgG3 hinge domain

Amino Acids 306 - 426 : VH region anti B7.1 Mab

Amino Acids 427 - 441 : (G<sub>4</sub>S)<sub>3</sub> flexible linker

Amino Acids 442 - 550 : VL region anti B7.1 Mab

5 Amino Acids 551 - 556 : His6 Tag

Figure 19 shows the DNA sequence of the dimerisation domain HDH

Nucleotides 1 - 33 : Human IgG3 hinge region

Nucleotides 34 - 82 : helix-domain

10 Nucleotides 83 - 90 : turn

Nucleotides 91 - 139 : helix-domain

Nucleotides 140 - 171 : Human IgG3 hinge region

Figure 20 shows the Protein sequence of the dimerisation domain HDH

15 Amino Acids 1 - 11 : Human IgG3 hinge region

Amino Acids 12 - 27 : helix-domain

Amino Acids 28 - 31 : turn

Amino Acids 32 - 46 : helix - domain

Amino Acid 47 - 57 : Human IgG3 hinge region

20

Figure 21 shows the DNA sequence of the dimerisation domain JEM-1

Nucleotides 1- 99 : JEM-1 dimerisation domain

Figure 22 shows the Protein sequence of the dimerisation domain JEM-1

25 Amino Acids 1- 33 : JEM-1 dimerisation domain

Figure 23 shows DNA sequence of monospecific Diabody B7-24: VH-B7-24/5/VL-B7-24/H6

Nucleotides 1-72 : pelB signal sequence

30 Nucleotides 73 - 414 : VH region anti B7.1 Mab (B7-24)

Nucleotides 415 -429 : G<sub>4</sub>S flexible linker

Nucleotides 430 - 756 : VL region anti B7.1 Mab (B7-24)

Nucleotides 757 - 773 : His6 Tag

Figure 24 shows Protein Sequence of monospecific Diabody B7-24: VH-B7-24/5/VL-  
5 B7-24/H6

Amino Acids 1- 24: pelB signal sequence

Amino Acids 25 - 138 : VH region anti B7.1 Mab

Amino Acids 139 - 143 : G<sub>4</sub>S flexible linker

Amino Acids 144 - 252 : VL region anti B7.1 Mab

10 Amino Acids 253 - 259: His6 Tag

Figure 25 shows DNA sequence of monospecific Diabody 1G10: VH-1G10/5/VL-  
1G10/H6

Nucleotides 1-72 : pelB signal sequence

15 Nucleotides 73 - 433 : VH region anti B7.2 Mab (1G10)

Nucleotides 434- 447 : G<sub>4</sub>S flexible linker

Nucleotides 448 - 786 : VL region anti B7.2 Mab (1G10)

Nucleotides 787 - 804 : His6 Tag

20 Figure 26 shows Protein Sequence of monospecific Diabody 1G10: VH-1G10/5/VL-  
1G10/H6

Amino Acids 1- 24 : pelB signal sequence

Amino Acids 25 -144 : VH region anti B7.2 Mab

Amino Acids 145 - 149 : G<sub>4</sub>S flexible linker

25 Amino Acids 150 - 262 : VL region anti B7.2 Mab

Amino Acids 263 - 268 : His6 Tag

Figure 27 shows DNA sequence of bispecific Diabody I : VH-1G10/5/VL-B7-24/H6

Nucleotides 1-117 : g3p - signal sequence

30 Nucleotides 118 - 483 : VH region anti B7.2 Mab (1G10)

Nucleotides 484 - 498 : G<sub>4</sub>S flexible linker

Nucleotides 499 - 825 : VL region anti B7.1 Mab (B7-24)

Nucleotides 826 - 843 : His6 Tag

Figure 28 shows the Protein sequence of bispecific Diabody I : VH-1G10/5/VL-B7-

5 24/H6

Amino Acids 1- 39 : g3p - signal sequence

Amino Acids 40 - 161: VH region anti B7.2 Mab

Amino Acids 162 - 166: G<sub>4</sub>S flexible linker

Amino Acids 167 - 275: VL region anti B7.1 Mab

10 Amino Acids 276 - 281: His6 Tag

Figure 29 shows the DNA sequence of bispecific Diabody II : VH-B7-24/5/VL-1G10

Nucleotides 1-117 : g3p - signal sequence

Nucleotides 118 - 465 : VH region anti B7.1 Mab (B7-24)

15 Nucleotides 466 - 480 : G<sub>4</sub>S flexible linker

Nucleotides 481 - 819 : VL region anti B7.2 Mab (1G10)

Figure 30 shows the Protein sequence of bispecific Diabody II : VH-B7-24/5/VL-1G10

Amino Acids 1- 39 : g3p - signal sequence

20 Amino Acids 40 - 155: VH region anti B7.1 Mab

Amino Acids 156 - 160: G<sub>4</sub>S flexible linker

Amino Acids 161 - 273: VL region anti B7.2 Mab

Figure 31 shows the DNA sequence of monospecific Triabody B7-24 : VH-B7-

25 24/0/VL-B7-24H6

Nucleotides 1-72 : pelB signal sequence

Nucleotides 73 - 414 : VH region anti B7.1 Mab (B7-24)

Nucleotides 415 - 741 : VL region anti B7.1 Mab (B7-24)

Nucleotides 742 - 759 : His6 Tag

30

Figure 32 shows the Protein sequence of monospecific Triabody B7-24 : VH-B7-

24/0/VL-B7-24H6

Amino Acids 1- 24 : pelB signal sequence

Amino Acids 25 - 138 : VH region anti B7.1 Mab

Amino Acids 139 - 247 : VL region anti B7.1 Mab

5 Amino Acids 248 - 253 : His6 Tag

Figure 33 shows the DNA sequence of monospecific Triabody 1G10 : VH-1G10/0/VL-1G10H6

Nucleotides 1-72 : pelB signal sequence

10 Nucleotides 72 - 433 : VH region anti B7.2 Mab (1G10)

Nucleotides 434 - 771 : VL region anti B7.2 Mab (1G10)

Nucleotides 772 - 789 : His6 Tag

Figure 34 shows the Protein sequence of monospecific Triabody 1G10 : VH-1G10/0/VL-1G10H6

15

Amino Acids 1- 24 : pelB signal sequence

Amino Acids 25 - 144 : VH region anti B7.2 Mab

Amino Acids 145 - 257 : VL region anti B7.2 Mab

Amino Acids 258 - 263 : His6 Tag

20

Figure 35 shows the DNA sequence of bispecific Triabody I : VH-1G10/0/VL-B7-24/H6

Nucleotides 1-117 : g3p - signal sequence

Nucleotides 118 - 483 : VH region anti B7.2 Mab (1G10)

25 Nucleotides 484 - 810 : VL region anti B7.1 Mab (B7-24)

Nucleotides 811 - 828 : His6 Tag

Figure 36 shows the Protein sequence of bispecific Triabody I : VH-1G10/0/VL-B7-24/H6

30 Amino Acids 1- 39 : g3p - signal sequence

Amino Acids 40 - 161 : VH region anti B7.2 Mab



Amino Acids 162 - 270: VL region anti B7.1 Mab

Amino Acids 271 - 276: His6 Tag

Figure 37 shows the DNA sequence of bispecific Triabody II : VH-B7-24/0/VL-1G10

5 Nucleotides 1-117 : g3p - signal sequence

Nucleotides 118 - 465 : VH region anti B7.1 Mab (B7-24)

Nucleotides 466 - 804 : VL region anti B7.2 Mab (1G10)

Figure 38 shows the Protein sequence of bispecific Triabody II : VH-B7-24/0/VL-1G10

10 Amino Acids 1- 39 : g3p - signal sequence

Amino Acids 40 - 155: VH region anti B7.1 Mab

Amino Acids 156 - 268: VL region anti B7.2 Mab

Figure 39 shows the gel filtration profile of scFv B7-24 L0 (a), scFv B7-24 L5 (b) and

15 scFv B7-24 L15 (c).

Figure 40 shows the binding of unpurified scFv B7-24, B7-24 diabodies (scFv B7-24 L5)- and B7-24 triabodies (scFv B7-24 L0) on B7.1 ED fusion protein.

20 Figure 41 shows the binding of unpurified scFv 1G10, 1G10 diabodies (scFv 1G10 L5) and 1G10 triabodies (scFv 1G10L0) on B7.2ED fusion proteins

Figure 42 shows binding of unpurified scFv B7-24, B7-24 diabodies (scFv B7-24 L5)- and B7-24 triabodies (scFv B7-24 L0) on different 3T6 cells.

25

Figure 43 shows binding of semi-purified scFv B7-24, B7-24 diabodies (scFv B7-24 L5)- and B7-24 triabodies (scFv B7-24 L0) on B7.1 ED fusion protein

Figure 44 shows binding of semi-purified scFv B7-24, B7-24 diabodies (scFv B7-24 L5)- and B7-24 triabodies (scFv B7-24 L0) on RPMI8866 cells

30

Figure 45 shows the neutralizing activity of scFv B7-24 and B7-24 diabodies (scFv B7-24 L5) in a MLR

5 Figure 46 shows the neutralizing activity of scFv B7-24 and B7-24 triabodies (scFv B7-24 L0) in a MLR

Figure 47 shows binding of B7-24 diabodies (scFv B7-24 L5)- and B7-24 triabodies (scFv B7-24 L0) gelfiltration fractions on B7.1ED fusion protein.

10 Figure 48 shows the gelfiltration of B7-24/1G 10 crosslinked monoclonal antibodies.

Figure 49 shows the binding of the crosslinked monoclonal antibodies on 3T6 cells.

15 Figure 50 shows the Biacore results of the crosslinked b7-24/1G10 monoclonal antibodies.

### DETAILED DESCRIPTION OF THE INVENTION

20 The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All of these publications and applications, cited previously or below are hereby incorporated by reference.

25 The present invention is based on the finding that molecules which cross-link, or cross-react with, B7.1 and B7.2, and which do not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28 efficiently inhibit antigen-specific T cell activation. Accordingly, these molecules can be used to prevent or treat (terms used interchangeably) diseases, such as transplant rejection, GVHD, allergy and autoimmune diseases, where antigen-specific T cell activation is pathogenic.

30 More particularly, the present invention relates to a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable

domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28, resulting in the inhibition of antigen-specific T cell activation.

The expression "molecule which cross-reacts with B7.1 and B7.2 and which does not comprise the extracellular domain of CTLA-4 or CD28" refers to any molecule known in the art which simultaneously binds (not necessarily to a common epitope of B.1 and B7.2) or binds to a common epitope of B7.1 (described by Freeman et al., 1989) and B7.2 (described by Freeman et al., 1993 and Azuma et al., 1993) and which does not comprise the extracellular domain of CTLA-4 or CD28. Molecules comprising the extracellular domains of CTLA-4 or CD28, such as CTLA-4Ig or CD28Ig respectively, are known to be able to cross-link, or crossreact with, B7.1 and B7.2 (described in US patent N° 5,434,131 and 5,521,288 to Linsley et al., respectively). However, we already indicated above why it is preferable not to use CTLA-4Ig as a therapeutic. Also CD28Ig was proven to be a very poor inhibitor of T-cell activation. Furthermore, the term "a molecule which cross-links B7.1 and B7.2" indicates that both B7.1 and B7.2 are physically bridged or connected by said molecule. It should also be clear that both B7 molecules which are connected by said molecule can be expressed on the same cell or on different cells. In this regard, molecules with a rigid structure such as diabodies, triabodies, small antigen binding peptides and low-molecular weight nonpeptide molecules will crosslink B7 molecules on different cells and may not be able to crosslink B7 molecules expressed on the same cell, whereas molecules with a flexible structure such as tetravalent antibodies will crosslink B7 molecules expressed on both the same and different cells. Furthermore, the expression "crosslinking" may also imply that the B7 molecules on the B7-expressing cells are not only physically bridged but that the cross-linking of both B7 molecules results in a biological effect on the B7-expressing cells. The latter biological effect may comprise the inhibition of synthesis of immunoactivating soluble mediators such as interleukin-12 (IL-12), interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF- $\alpha$ ) by the B7-expressing cell or the activation of synthesis of immunosuppressive mediators such as interleukin-10 (IL-10), tumor growth factor-beta (TGF- $\beta$ ) and prostaglandins by the B7-expressing cell, or any other known biological effect

which, preferably, favours the inhibition of T cell activation. The terms "a variable domain of a monkey antibody" specifically refer to the variable domains of the macaque monoclonal antibodies 7B6, 16C10, 7C10 and 20C9 described in WO 96/40878 (PCT/US96/10053) to Anderson et al. The latter reference is incorporated  
5 by reference in its entirety herein.

More specifically, the invention relates to a molecule which comprises at least one first domain which binds B7.1 or B7.2 or cross-reacts with B7.1 and B7.2, at least one second domain which binds B7.1 or B7.2 or cross-reacts with B7.1 and B7.2, and optionally a third domain which couples the first and the second  
10 domain(s). The term domain refers to any antibody-like polypeptide, any nonantibody-like peptide or any nonpeptide organic molecule. Examples of such molecules are diabodies which bind in a monovalent fashion to B7.1 and B7.2 or which bind in a bivalent fashion to a common epitope of B7.1 and B7.2 (hereafter termed B7.12), triabodies which bind simultaneously in a monovalent fashion to  
15 B7.1 and bivalently to B7.2 or in a monovalent fashion to B7.2 and bivalently to B7.1 or trivalently to B7.12, tetravalent antibodies which bind bivalently to B7.1 and B7.2 or tetravalently to B7.12, and small antigen binding peptides or low molecular weight nonpeptide molecules which bind mono-or multivalently to B7.1, B7.2 and/or B7.12. It should be clear that any other possible combination, for example a  
20 tetravalent antibody which binds bivalently to B7.12 and bivalently to B7.2, is also part of the present invention.

As used herein, the term "any chemical coupling agent or any oligomerization domain" refers to any molecule known in the art which is capable of coupling the said first and second domains to each other. Examples of such  
25 domains are the known leucine zipper of c-fos and c-jun (Kostelny et al., 1992; de Kruif & Logtenberg, 1996), the polyglutamic acid-polylysine domains as described in US Patent N° 5,582,996 to Curtis, the helix-turn-helix motif described by Pack et al. (1993) and the max-interacting proteins and related molecules as described in US Patent N° 5,512,473 to Brent and Zervos. The term "optionally" as used in the  
30 terms "optionally a third domain which couples the first and the second domain(s)" indicates that a third domain can, but does not have to, be part of a molecule of the

present invention as described above

As used herein, the term "antibody" refers to polyclonal or monoclonal antibodies. The term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made.

As used herein, the term "humanized antibody" means that at least a portion of the framework regions of an immunoglobulin are derived from human immunoglobulin sequences.

As used herein, the term "single chain antibody" refers to antibodies prepared by determining the binding domains (both heavy and light chains) of a binding antibody, and supplying a linking moiety which permits preservation of the binding function. This forms, in essence, a radically abbreviated antibody, having only that part of the variable domain necessary for binding the antigen.

Determination and construction of single chain antibodies are described in U.S. Patent No. 4,946,778 to Ladner et al.

As used herein, the term "fragments (of antibodies)" refers to  $F_{ab}$ ,  $F_{(ab)2}$ ,  $F_v$ , and other fragments which retain the antigen binding function and specificity of the parent antibody.

Antibodies to human B7.1 and human B7.2 are known in the art. The present invention contemplates a new use for such antibodies as detailed above.

Monoclonal antibody B7-24 was prepared as described in the international application WO 94/01547.

Monoclonal antibodies 5B5 and 1G10 were prepared essentially as described in U.S. Patent No. 5,397,703 or international application WO 94/01547 and can be obtained at Innogenetics N.V., Industriepark Zwijnaarde 7, box 4, B-9052 Ghent, Belgium, fax +32 9 241 07 99.

Monoclonal antibodies 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3 and 14F1 were prepared as described in example 1 (see further) and can be obtained at Innogenetics N.V., Industriepark Zwijnaarde 7, box 4, B-9052 Ghent, Belgium, fax +32 9 241 07 99.

The present invention more specifically relates to miniantibodies, diabodies, triabodies, tetravalent antibodies, antigen-binding peptides and low molecular weight nonpeptide molecules which cross-link, or cross-react with, B7.1 and B7.2, and do not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28, and which are produced by the following methods:

1) chemical linkage of anti-B7.1 and anti-B7.2 antibodies or univalent fragments thereof following a method as described by Fanger et al. (1992). See also the *Examples* section further in the present application.

2) genetically engineering of non-covalently-linked miniantibodies as described by Pack et al. (1993), diabodies as described by Holliger et al. (1993) and tetravalent antibodies as described by Pack et al. (1995). See also the *Examples* section further in the present application.

3) genetically engineering of covalently-linked chelating recombinant antibodies as described by Kranz et al. (1995), single chain antibodies fused to protein A or Streptavidin as described by Ito and Kurosawa (1993) and Kipriyanov et al. (1996) and bispecific tetravalent antibodies as described in EP 0 517 024 to Bosslet and Seeman, and Coloma and Morrison (1997). See also the *Examples* section further in the present application.

4) genetically engineering of triabodies as described by Kortt et al (1997) and as given in the *Examples* section of the present application.

5) phage display of Ab combinatorial libraries resulting in the production of high-affinity antibodies and screening of random DNA sequence phage display libraries for small antigen-binding peptides as described in US patent numbers 5,403,484 and 5,571,698 and 5,223,409 to Ladner et al., Schultz and Schultz (1996), Parsons et al. (1996), McGuinness et al. (1996) and Hoogenboom (1997) and Georgiou et al. (1997). See also the *Examples* section further in the present application.

6) generation of hybridomas, derived from immunized transgenic mice, containing large sections of the human immunoglobulin (Ig) gene loci in the germ line, integrated by the yeast artificial chromosomal (YAC) technology, resulting in effective blocking antibodies as described by Mendez et al (1997).

7) rational drug design resulting in the production of low-molecular weight

nonpeptide molecules as described by Wiley and Rich (1993), Wendolowski et al. (1993) and Lybrand (1995).

8) 'High Throughput Screening' (HTS) of chemical or natural libraries, resulting in the production of peptides or non-peptides as described by Sarubbi et al. (1996).

5 The latter references are incorporated in their entirety herein.

As used herein, the term "small antigen-binding peptides or fragments" refers to any peptide (i.e. a polymer composed of at least two amino acids) which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28. The term "low-molecular weight nonpeptide molecules" refers to any molecule which is not a peptide and which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28.

The present invention further relates to a composition comprising a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28 in a pharmaceutically acceptable excipient, possibly in combination with immunosuppressive agents such as cyclosporin A (Sandimmune, Neoral; Sandoz-Sangstat), FK 506 (Tacrolimus, Prograf; Fujisawa), rapamycin (Sirolimus; American Home Products), OKT-3 (anti-CD3 mAb; Johnson & Johnson), OKT-4 (anti-CD4 mAb; Johnson & Johnson), SB-210396 (anti-CD4 mAb; Smithkline Beecham), T10B9 (anti-TcR antibody; MedImmune), BTI 322 (anti-CD2 mAb; Biotransplant), Mycophenolate mofetil (Cellcept; Roche), anti-thymocyte immunoglobulin (Thymoglobulin (rabbit); Pasteur Mérieux), anti-lymphocyte immunoglobulin (Lymphoglobulin (equine); Pasteur Mérieux), anti-lymphocyte immunoglobulin (ATG Fresenius (rabbit); Hoechst Marion Roussel), azathioprine (Imuran; Glaxo Wellcome), leflunomide (Hoechst Marion Roussel), triple therapy combining cyclosporin A (Sandimmune, Neoral; Sandoz-Sangstat) with azathioprine (Imuran; Glaxo Wellcome) and glucocorticosteroids, adenosin deaminase inhibitor (Pentostatin; Warner-Lambert), purine nucleoside phosphorylase (PNP) inhibitor (Peldistine; Biocryst Pharmaceuticals), MHC-peptide

(Allotrap-2702; Sangstat) and IL-2 receptor mAb (Pasteur-Mérieux, Lederle/American Home products, Protein Design Labs, for use as a medicament to prevent allograft rejection, GVHD, allergy and autoimmune diseases.

As used herein, the term "composition" refers to any composition comprising  
5 as an active ingredient a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28 according to the present invention possibly in the presence of suitable excipients known to the skilled man. The molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not  
10 comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28 of the invention may thus be administered in the form of any suitable composition as detailed below by any suitable method of administration within the knowledge of a skilled man. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be  
15 formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable excipient. Such excipients are inherently nontoxic and nontherapeutic. Examples of such excipients are saline, Ringer's solution, dextrose solution and Hank's solution. Nonaqueous excipients such as fixed oils and ethyl oleate may also be used. A  
20 preferred excipient is 5% dextrose in saline. The excipient may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

The molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the  
25 extracellular domain of CTLA-4 or CD28 of the invention are administered at a concentration that is therapeutically effective to prevent allograft rejection, GVHD, allergy and autoimmune diseases. The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that the molecule cross-links, or cross-reacts with, B7.1 and B7.2, and which does not  
30 comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28, is given at a dose between 1  $\mu$ g/kg and 10 mg/kg, more preferably



between 10  $\mu\text{g/kg}$  and 5 mg/kg, most preferably between 0.1 and 2 mg/kg.

Preferably, it is given as a bolus dose. Continuous short time infusion (during 30 minutes) may also be used. If so, the molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28 constructs or compositions comprising the same may be infused at a dose between 5 and 20  $\mu\text{g/kg/minute}$ , more preferably between 7 and 15  $\mu\text{g/kg/minute}$ .

According to the specific case, the "therapeutically effective amount" of a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28 needed should be determined as being the amount sufficient to cure the patient in need of treatment or at least to partially arrest the disease and its complications. Amounts effective for such use will depend on the severity of the disease and the general state of the patient's health. Single or multiple administrations may be required depending on the dosage and frequency as required and tolerated by the patient.

With regard to the use of a molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA-4 or CD28 to prevent allograft rejection, it should be stressed that molecules of the present invention or the compositions comprising the same may be administered before, during or after the organ transplantation as is desired from case to case. In case the molecules or the compositions comprising the same are administered directly to the host, treatment will preferably start at the time of the transplantation and continue afterwards in order to prevent the activation and differentiation of host T cells against the MHC on the allograft. In case the donor organ is ex vivo perfused with molecules or the compositions comprising the same, treatment of the donor organ ex vivo will start before the time of the transplantation of the donor organ in order to prevent the activation and differentiation of host T cells against the MHC on the allograft.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it

should be noted that these embodiments are illustrative and are not to be construed as restricting the invention in any way.

## EXAMPLES

5

### *1. Making monoclonal Antibodies to B7.1 and B7.2*

#### *1.1 Making monoclonal Antibodies to B7.1*

10 One female BALB/c mice was immunized (injected intraperitoneally) four times (i.e., at days 0, 13, 26 and 213) with  $5 \times 10^6$  Sf9 insect cells that were infected with a recombinant baculovirus containing a human B7.1 cDNA. Three days after the last injection, spleen cells were retrieved from the immunized mice and used for cell fusion mainly according the procedure as described by Köhler and Milstein  
15 (1975). To this end, dissociated splenocytes from the immunized mice were fused with murine myeloma cells SP2/0-Ag14 (ATCC, CRL-1581) at a ratio of 10:3 using a polyethylene glycol/DMSO solution. The fused cells were mixed up and resuspended in DMEM medium supplemented with hypoxanthine, thymidine, sodium pyruvate, glutamine, a non-essential amino acid solution, 10% inactivated  
20 fetal calf serum, 10% inactivated horse serum and 10% BM-Conditioned. The cells were then distributed to 960 wells on tissue culture plates to which aminopterin was added 24 hours after the cell fusion. Each well contained between 1 to 5 growing hybridoma clones at the average. After eight days supernatants of the 960 wells were combined in groups of 10 for primary screening. The 96 pools were screened  
25 for the presence of specific antibody by FACS analysis using the B7.1 positive Epstein-Barr virus (EBV)-transformed human B cell line, ARC. For this, cells ( $5 \times 10^5$  cells/sample) were incubated for 30' at 4°C with the different (100 ml undiluted) supernatants. After washing of the cells with PBS supplemented with 2% inactivated FCS and 0.02% azide, the cells were incubated another 30' at 4°C with  
30 goat anti-mouse antibodies conjugated to fluoresceine isothiocyanate (FITC). Cells were washed twice in PBS supplemented with 2% inactivated FCS and 0.02%

azide, fixed using 0.5% paraformaldehyde and analyzed with a FACScan flow cytometer (Becton Dickinson). The specific binding of the monoclonal antibodies is expressed as the mean fluorescence intensity in arbitrary units. The first screening yielded 3 positive pools. The thirty wells corresponding to the three positive pools were subjected to a second screening with the FACS screening technique described above using ARC cells ( $2.5 \times 10^5$  cells/sample). This second screening provided one individual positive well containing antibodies reactive with the B7.1-expressing ARC cells. This positive well was subcloned and one stable hybridoma clone named 5B5 was obtained. This hybridoma clone secretes mouse antibodies of the IgG3 isotype.

The antibody secreted by the hybridoma clone 5B5 was tested for specific binding to the human B7.1 molecule in a FACS experiment using the B7.1-expressing ARC cells or the mouse fibroblasts, 3T6 cells, transfected with cDNA encoding human B7.1 molecule (3T6-B7.1 cells) (De boer et al., 1992). As control non-transfected 3T6 cells were used. Cells ( $2.5 \times 10^5$  cells/sample) were incubated with the supernatant of the hybridoma clone 5B5 for 30 min. at  $4^\circ\text{C}$ . Thereafter, the cells were washed (PBS supplemented with 2% inactivated FCS and 0.02% azide) three times and incubated with FITC-labeled goat anti-mouse antiserum (i.e. GAM-FITC). The cells were also incubated with the GAM-FITC alone. After another 3 washes, the cells were analysed for fluorescent staining using a FACScan instrument. Results showed that the antibodies secreted by the 5B5 hybridoma clones specifically bound to the B7.1-expressing ARC and 3T6/B7.1 cells whereas 3T6 cells that do not express B7.1, did not exhibit any binding significantly greater than that of the control monoclonal antibody or GAM-FITC.

Moreover, specific binding of the antibodies secreted by 5B5 hybridoma clones was further analyzed in a competition experiment. To this end, ARC cells ( $5 \times 10^5$  cells/staining) were incubated with the supernatant of the 5B5 hybridoma clones together with 1 mg of biotinylated anti-B7.1 monoclonal antibody (B7-24) for 30' at  $4^\circ\text{C}$ . Thereafter, cells were separated from the supernatant, washed and incubated for 30' at  $4^\circ\text{C}$  with FITC-labeled streptavidin. Results showed that complete inhibition of binding of the anti-B7.1 monoclonal antibody (B7-24) could be

obtained with 5B5 hybridoma clones.

Moreover, the antibodies, secreted by the 5B5 hybridoma clone were tested for their capacity to inhibit the proliferation of human peripheral blood T lymphocytes, activated with anti-CD3 (OKT-3) in the presence of the mouse 3T6 cells transfected with the cDNA encoding human B7.1 molecule and human CD32 (3T6/CD32/B7.1) (deBoer et al., 1992). Human peripheral blood T lymphocytes were isolated from buffy coat by density centrifugation. T cells were further purified by cold aggregation of the monocytes ( $4 \times 10^6$  PBMC/ml in RPMI bic + 10 % iFCS, 30' rotation at 4 °C; 15' on ice to separate the monocyte aggregates; supernatant contains the enriched T cells). T cells were further enriched by depletion of monocytes, B cells and NK cells using Lympho-Kwik T (One Lambda, Los Angeles, CA) according to the manufacturers protocol. Mitomycin C-treated 3T6/CD32/B7.1 cells (at 200  $\mu$ l Mitomycin C at 250  $\mu$ g/ml to 800  $\mu$ l RPMI bic + 10 % iFCS during 45' at 37°C, washed twice in RPMI bic + 10 % iFCS) at  $10^4$  cells /well were incubated with anti-CD3 mAb (OKT3, 0.5 mg/ml) for 1 h at 37°C followed by a 1 h incubation at 37°C with decreasing concentrations of the antibodies secreted by the 5B5 hybridoma clone. Subsequently purified T cells ( $5 \times 10^4$  cells/well) were added and incubated for 5 days. After 5 days of culture, the cells were pulsed for 6 to 8 h with 1  $\mu$ Ci [ $^3$ H]-Thymidine, after which the cells were harvested using an automated cell harvester. [ $^3$ H]-Thymidine incorporation was determined with a liquid scintillation counter. Proliferation of T cells was performed in triplicate wells. Results showed that the proliferation of the T cells was inhibited by the antibodies secreted by the 5B5 hybridoma clones. Thus, the antibodies secreted by the 5B5 hybridoma clone are neutralizing antibodies.

## 1.2. Making monoclonal Antibodies to B7.2

### 1.2.1 Fusion 1

Two female BALB/c mice were immunized (injected intraperitoneally) four times (i.e., at days 0, 28, 56 and 208) with Sf9 insect cells that were infected with a recombinant baculovirus containing a human B7.2 cDNA. Three days after the last

injection, spleen cells were retrieved from the immunized mice and used for cell fusion mainly according to the procedure as described by Kohler and Milstein (1975). Dissociated splenocytes from the immunized mice were fused with SP2/0 murine myeloma cells at a ratio of 10:3 using a polyethylene glycol/DMSO solution.

5 The fused cells, derived from the two mice, were mixed up and resuspended in DMEM medium supplemented with hypoxanthine, thymidine, sodium pyruvate, glutamine, a non-essential amino acid solution, 10% inactivated fetal calf serum and 10% inactivated horse serum. The cells were then distributed to 1440 wells on tissue culture plates to which aminopterin was added 24 hours after the cell fusion.

10 Each well contained between 1 to 5 growing hybridoma clones at the average.

After ten days, supernatants from the 1440 primary wells were combined in groups of five to form 288 pools for primary screening. The 288 pools were screened for the presence of specific antibody by FACS analysis using the B7.2 positive Epstein-Barr virus (EBV) transformed human B cell line, RPMI 8866. For this, cells ( $0.5 \times 10^5$

15 cells/sample) were incubated for 15' at 4°C with the different supernatants (100  $\mu$ l undiluted). After washing twice in RPMI1640 supplemented with 10% FCS, the cells were incubated for another 15' at 4°C with goat anti-mouse antibodies conjugated to fluorescein isothiocyanate (FITC). The cells were washed twice in RPMI1640 supplemented with 10% FCS and finally suspended in PBS supplemented with 1% BSA and 0.1% NaN<sub>3</sub> and analyzed with a FACScan flow cytometer (Becton Dickinson).

20 The specific binding of the monoclonal antibodies is expressed as the mean fluorescent intensity in arbitrary units. This first screening yielded eighteen positive pools. The ninety wells corresponding to the eighteen positive pools were

subjected to a second screening, with the FACS screening technique described

25 above using the B7.2-expressing human EBV-transformed B cell line RPMI 6688.

This second screening provided eleven individual positive wells containing antibodies reactive with the B7.2-expressing EBV-transformed human B cell line RPMI 8866. These positive wells were subcloned and eight stable hybridoma clones named 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1 were obtained. These

30 hybridoma clones secrete mouse antibodies of different IgG isotypes: IgG1: 9D8; IgG2 $\alpha$ : 5F3, 7B8, 14F1; IgG2b: 3H10, 11B9, 13B9, 13D3.

The antibodies secreted by hybridoma clones 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1 were tested for specific binding to the human B7.2 molecule. B7.2-expressing EBV-transformed human B cells (RPMI 8866), freshly isolated peripheral blood human T cells and monocytes were incubated with the supernatant of the different hybridoma clones 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1 or an isotype matched control monoclonal antibody (control mAb) for 30 min. at 4°C. Thereafter, the cells were separated from the supernatant, washed three times and incubated with FITC-labeled goat anti-mouse IgG antiserum (i.e. GAM-FITC). The cells were also incubated with the GAM-FITC alone. After another 3 washes, the cells were analysed for fluorescent staining using a FACScan instrument. Results showed that the antibodies secreted by the 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1 hybridoma clones specifically bound to the B7.2-expressing RPMI 8866 cells and the B7.2 expressing human monocytes, whereas freshly isolated human peripheral blood T cells that do not express B7.2, did not exhibit any binding significantly greater than that of the control monoclonal antibody or GAM-FITC. In another experiment, mouse 3T6 cells transfected with the cDNA encoding human B7.1 molecule (3T6-B7.1) or encoding the human B7.2 molecule (3T6-B7.2) (de Boer et al., 1992) were used to demonstrate that the antibodies secreted by hybridoma clones 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1 are specific for B7.2. Results showed that the antibodies secreted by the hybridoma clones 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1 specifically bound to the B7.2-expressing 3T6 cells (3T6-B7.2), whereas 3T6 cells expressing the B7.1 (3T6-B7.1) molecule did not exhibit any binding significantly greater than that of the GAM-FITC. Moreover, the antibodies, secreted by the different hybridoma clones 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1, were tested for their capacity to inhibit the proliferation of human peripheral blood T lymphocytes, activated with anti-CD3 (OKT-3) in the presence of the B7.2 expressing EBV transformed B cell line RPMI 8866. Human peripheral blood T lymphocytes were isolated from buffy coat by density centrifugation (Ficoll Pacque, density 1.077). T cells were further purified by cold aggregation of the monocytes (4X 10<sup>6</sup> PBMC/mln RPMI bic + 10 % iFCS, 30' rotation at 4 °C; 15' on ice to separate the monocyte aggregates; supernatant contains the enriched T cells). T cells were

further enriched by depletion of monocytes, B cells and NK cells using Lympho-Kwik T (One Lambda, Los Angeles, CA) according to the manufacturers protocol. The purified T cells proliferated in the presence of anti-CD3 mAb (OKT-3, 0.5 µg/ml) and Mitomycin C treated RPMI8866 cells (at 200 µl Mitomycin C at 250 µg/ml to 800 µl RPMI bic + 10 iFCS during 45' at 37°C, wash twice in RPMI bic + 10 % iFCS). Decreasing concentrations of the antibodies, secreted by the 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1 hybridoma clones were added to the T cell cultures. After 5 days of culture, the cells were pulsed for 6 to 8 h with 1 µCi [<sup>3</sup>H]-Thymidine, after which the cells were harvested using an automated cell harvester. [<sup>3</sup>H]-Thymidine incorporation was determined with a liquid scintillation counter. Proliferation of T cells was performed in quadruplicate wells. Results showed that the proliferation of the T cells was inhibited by the antibodies, secreted by the different hybridoma clones 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1. Thus, the antibodies, secreted by the different hybridoma clones 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3, 14F1 are neutralizing antibodies.

#### 1.2.2. Fusion 2

One female BALB/c mice was immunized (injected intraperitoneally) four times (i.e., at days 0, 27, 56 and 97) with 5x10<sup>6</sup> Sf9 insect cells that were infected with a recombinant baculovirus containing a human B7.2 cDNA. Three days after the last injection, spleen cells were retrieved from the immunized mice and used for cell fusion mainly according the procedure as described by Köhler and Milstein (1975). Dissociated splenocytes from the immunized mice were fused with SP2/0 murine myeloma cells at a ratio of 10:3 using a polyethylene glycol/DMSO solution. The fused cells were mixed up and resuspended in DMEM medium supplemented with hypoxanthine, thymidine, sodium pyruvate, glutamine, a non-essential amino acid solution, 10% inactivated fetal calf serum, 10% inactivated horse serum and 10% BM-Condimed. The cells were then distributed to 960 wells on tissue culture plates to which aminopterin was added 24 hours after the cell fusion. Each well contained between 1 to 5 growing hybridoma clones at the average. After eight

days supernatants of the 960 wells were combined in groups of 5 for primary screening. The 192 pools were screened for the presence of specific antibody by FACS analysis using freshly isolated monocytes. Monocytes were isolated from buffy coat on Ficoll-Paque (density gradient 1.077; Pharmacia) gradients. After three washings, monocytes were removed by cold agglutination (Mentzer et al., 1986). A 15 ml tube containing  $50 \times 10^6$  PBMC in 10 ml of culture medium was slowly rotated for 30 minutes at 4°C. Monocyte aggregates were allowed to sediment over a 15 minute period, and the non aggregated cells were carefully aspirated. Monocytes ( $5 \times 10^5$  cells/sample) were incubated for 30' at 4°C in PBS supplemented with 2% FCS, 0.02% sodium azide and 10% normal rabbit serum with the different (100 ml undiluted) supernatants. Thereafter the cells were washed two times with PBS supplemented with 2% inactivated FCS and 0.02% sodium azide and incubated with goat anti-mouse (IgG and IgM) antibodies conjugated to fluorescein isothiocyanate (FITC) for 30' at 4°C. After another two washes, cells were fixed using 0.5% paraformaldehyde and analyzed with a FACScan flow cytometer (Becton Dickinson). The specific binding of the monoclonal antibodies is expressed as the mean fluorescent intensity in arbitrary units. The first screening yielded five positive pools. The twenty-five wells corresponding to the five positive pools were subjected to a second screening with the FACS screening technique described above using  $5 \times 10^5$  freshly isolated monocytes for each staining. This second screening provided one individual positive well containing antibodies reactive with the monocytes. This positive well was subcloned and the subclones were subjected to a FACS screening as described above using the B7.2 expressing EBV-transformed human B cell line RMP18866, ( $5 \times 10^5$  cells/staining). Out of this subcloning one stable hybridoma clone named 1G10 was obtained. This hybridoma clone secretes mouse antibodies of the IgG2a isotype. The antibodies secreted by the hybridoma clone 1G10 were tested for specific binding to the human B7.2 molecule. To this end, FACS staining on mouse 3T6 cells, transfected with the cDNA encoding human B7.2 molecules (3T6/B7.2) and on B7.2-negative 3T6 cells was performed. The 3T6 and 3T6/B7.2 cells were firstly incubated for 20' at 4°C in PBS supplemented with 5% inactivated FCS and 0.02%



sodium azide and 10% normal rabbit serum. Subsequently, the cells were incubated with the supernatant of the hybridoma clone 1G10 for 30 min. at 4°C. Thereafter, the cells were separated from the supernatant, washed three times and incubated with fluorescein isothiocyanate-labeled goat anti-mouse antiserum (i.e. GAM-FITC). The cells were also incubated with the GAM-FITC alone. After another 3 washes, the cells were analysed for fluorescence staining using a FACScan flow cytometer (Becton Dickinson). Results showed that the antibodies secreted by the 1G10 hybridoma clones specifically bound to the B7.2-expressing 3T6/B7.2 cells whereas 3T6 cells that do not express B7.2, did not exhibit any binding significantly greater than that of the control monoclonal antibody or GAM-FITC.

Moreover, the antibodies, secreted by the 1G10 hybridoma clone were tested for their capacity to inhibit the proliferation of human peripheral blood T lymphocytes, activated with anti-CD3 (OKT-3) in the presence of the mouse 3T6 cells transfected with the cDNA encoding human B7.2 molecule and human CD32 (3T6/CD32/B7.2).

Human peripheral blood T lymphocytes were isolated from buffy coat by density centrifugation. T cells were further purified by cold aggregation of the monocytes ( $4 \times 10^6$  PBMC/ml in RPMI bic + 10 % iFCS, 30' rotation at 4 °C; 15' on ice; supernatant contains the enriched T cells). T cells were further enriched by

depletion of monocytes, B cells and NK cells using Lympho-Kwik T (One Lambda, Los Angeles, CA) according to the manufacturers protocol. Mitomycin C-treated 3T6/CD32/B7.2 cells (at 200  $\mu$ l Mitomycin C at 250  $\mu$ g/ml to 800  $\mu$ l RPMI bic + 10 iFCS during 45' at 37°C, wash twice in RPMI bic + 10 % iFCS) at  $10^4$  cells /well were incubated with anti-CD3 mAb (OKT3, 0.5 mg/ml) for 1 h at 37°C followed by a 1 h

incubation at 37°C with decreasing concentrations of the antibodies secreted by the 1G10 hybridoma clone. Subsequently purified T cells ( $5 \times 10^4$  cells/well) were added and incubated for 5 days. After 5 days of culture, the cells were pulsed for 6 to 8 h with 1  $\mu$ Ci [ $^3$ H]-Thymidine, after which the cells were harvested using an automated cell harvester. [ $^3$ H]-Thymidine incorporation was determined with a liquid scintillation counter. Proliferation of T cells was performed in triplicate wells.

Results showed that the proliferation of the T cells was inhibited by the antibodies secreted by the 1G10 hybridoma clones. Thus, the antibodies secreted by the 1G10

hybridoma clone are neutralizing antibodies.

## 2. Isolation of recombinant human antibodies that bind to human B7.1 or B7.2

5 A large naïve human phage displayed Fab repertoire (Target Quest, Maastricht, The Netherlands), which contains  $4.1 \times 10^{10}$  antibody molecules was screened for binding to human B7.1 or B7.2 molecules. The direct selection was performed on biotinylated B7.1 ED or B7.2ED fusion proteins (B7.1ED-Bio or B7.2ED-Bio) and specific phage antibodies captured by streptavidin paramagnetic particles and a magnet. As both, B7.1 and B7.2 are presented as immunoglobulin fusion proteins, we performed the selection in the presence of a 10 fold molar excess of human IgG to compete out the antibodies specific for the Fc moiety. Different (4) rounds of selection were performed. In the first round of selection the human Fab library was tested for binding 500 nM B7.1 ED-Bio, in the second and third round of selection the previous selected Fab's were tested for binding 100nM B7.1ED-Bio and in the fourth round of selection the previous selected Fab's were tested for binding 10 nM B7.1ED-Bio. In this approach the selection is for antibodies specific for the native conformation of the antigen. Specific clones are screened in a phage ELISA for binding to B7.1ED or B7.2ED fusion protein. The direct selection approach for hB7.1 binders resulted in 16 out of 40 clones that gave strong binding to B7.1ED fusion protein and not to B7.2ED fusion protein or human IgG using phage ELISA. In this phage ELISA, antigen (B7.1ED or B7.2 ED fusion protein) was coated at 5ug/ml in 100mM sodium hydrogen carbonate pH 9.6 in an ELISA plate and incubated overnight at 4°C. Wells were washed 2 times with both PBS and PBS tween-20 (0.1%) and then blocked for at least 30 minutes at room temperature with 2% Marval PBS. Plates were washed a further 3 times with PBS and PBS plus tween. To each well 50ul of 4% Marval PBS was added and 50ul of phage supernatants. These were mixed by pipetting up and down and left for 1.5 hrs. Plates were washed 3 times with PBS and PBS plus tween. A secondary antibody of goat anti immunoglobulin was added to each well and incubated for 1 hour at room temp. Antibody was discarded and washed 3 times with both PBS and PBS tween.

Positive phage antibodies were then detected using standard TMB staining and detection at OD 450. The reaction was stopped by adding 100ul of 2M H<sub>2</sub>SO<sub>4</sub>. Results showed that there was a high diversity of binders to B7-1Ig in the 3<sup>rd</sup> round of selection at 100nM. Nine different patterns were obtained. After the 4<sup>th</sup> round of selection on B7.1-Ig (10nM) one binder was recovered. This clone may have been selected for higher affinity by the 10 fold reduction in antigen concentration from round 3 to 4, however an increased phage display efficiency cannot be excluded as a contributing factor to the preferential selection of this clone.

The ten clones pCES-1Fab1 to 10 (1A, 1E, 2A, 2G, 3E, 3G, 3H, 4G, 5C, 5H), obtained by screening on B7.1ED fusion protein using the Phage Display Technology were transformed to JM83 expression strain. An overnight culture was 20x diluted in LB+ 100µg/ml amp+ 1% glucose and incubated at 28°C until an OD<sub>600</sub> of 0.5 was reached. After removing the glucose of the medium, the culture was induced with 0.1mM IPTG and further incubated at 28°C during ± 18h. To isolate soluble periplasmic proteins, the method described by Neu and Heppel (1965) was used. Briefly, cells were harvested by centrifugation and resuspended in ice cold shockbuffer (100mM Tris-HCl pH 7.4; 20% sucrose, 1mM EDTA, pH8). After incubation on ice during 10' with occasional stirring, the mixture was centrifuged at 10.000rpm during 1,5'. The supernatants was removed and the pellet was immediately resuspended in ice cold distilled water. After incubation on ice during 10' with occasional stirring, the mixture was centrifuged at 14.000rpm and the obtained supernatants was the soluble periplasmic fraction. A considerable amount was secreted into the periplasm and a small amount leaked in the supernatants.

The Fab's (1A, 1E, 2A, 2G, 3E, 3G, 3H, 4G, 5C, 5H) were tested in a ELISA experiment. In this ELISA, B7.1ED fusion protein ( 0.5 µg/ml) was directly coated on the plate (37°C, 2 hours) followed by blocking in PBS 0.1% casein for 1 hour at 37°C. After washing 5 times, wells were subsequently incubated with soluble Fab (1h, 37°C), washed 5 times and incubated (1h, 37°C) with HRP labeled mouse anti-human mAb (conc) followed by the addition of TMB as substrate. Plates were read at 450-595 nm on a microtiter plate reader. Results showed that the anti-B7.1 Fab molecules

bind and thus recognize to the B7.1ED fusion molecule. Human B7.2 binding FAb molecules are selected in a similar way using the B7.2 ED fusion proteins in the different selection rounds.

It is necessary to ensure the removal of antibodies specific for the Fc moiety. Therefore the selection is done in a similar way as previous described, using B7.1glu-glu or B7.2 his molecules; which do not have the Fc portion.

*3. Expression of B7.1 extracellular domain and B7.2 extracellular domain fusion proteins and expression of soluble B7.1 glu-glu and soluble B7.2 his molecules*

Soluble proteins are generated which comprise the extracellular domains (ED) of respectively human B7.1 and human B7.2 proteins carboxyterminally fused to the Fc-domain of a human IgG $\gamma$ 1 (further referred as ED fusion protein), the peptide EEEEEYMPME (glu-glu epitope) or a six histidine peptide (his) (further referred as ED-glu-glu or ED-his proteins).

All B7 proteins are expressed in insect cells using the recombinant baculovirus expression system (BEVS). Alternative expression systems are used. The soluble B7.1ED and B7.2ED fusion proteins, B7.1ED glu-glu and B7.2ED his proteins are needed for the further selection and optimization of higher affinity antibodies.

*3.1. Generation of recombinant baculovirions expressing soluble hB7.1ED or hB7.2 ED fusion proteins or hB7.1glu-glu or hB7.2 his proteins.*

Expression of a recombinant protein using BEVS is realised by integration of a foreign gene expression cassette, consisting of the protein-encoding cDNA sequence under transcriptional control of a strong baculoviral promoter (e.g. polyhedrin promoter), in the genome of the *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV), the prototype of the Baculoviridae, at a genome locus non-essential for in vitro replication of the virus (e.g. polyhedrin locus).

As the baculovirus genome is too large, easy insertion of this recombinant expression cassette (cDNA and viral promoter) by simple cloning techniques is not

possible. Therefore generation of the recombinant baculovirus genome was performed using a technology based on in vivo homologous recombination between the baculovirus wild type genome and a transfer plasmid containing the foreign expression cassette flanked by baculoviral genome sequences, both introduced in insect cells by classical cotransfection technology.

The resulting recombinant genome encodes a recombinant virus that is subsequently purified and amplified providing a high titre recombinant virus stock that can be used for large scale infection of insect cells thereby producing large amounts of the foreign protein.

### *3.1.1. Construction of baculo transfer plasmids for hB7.1ED Fc and human B7.2ED Fc recombinant baculovirus generation*

#### *3.1.1.1. Design of universal baculo transfer vector pVL-Fc*

Fusion proteins of both the hB7.1 and hB7.2 ED with the Fc part (Hinge-CH2-CH3) of a humanIgG $\gamma$ 1 immunoglobulin were expressed as recombinant baculovirus proteins. The cloning of the human IgG $\gamma$ 1-Fc sequence was performed by means of RT-PCR on mRNA isolated from stimulated human B cells ( 16h coculture with mitomycin C treated mouse fibroblast (3T6) transfected with cDNA encoding human CD40Ligand, 3T6/hCD40L, in the presence of 20U/ml rhIL2), using specific PCR-primers designed on the hIgG $\gamma$ 1 sequence published by Ellison et al.(1982, NAR :4071). The PCR amplified DNA fragment consisted of a 6 aminoacid encoding linker sequence including a BamHI site followed by the cDNA sequence coding for the complete Hinge-CH2-CH3 domain of the IgG $\gamma$ 1, and a stop codon. The amplified sequence was inserted in the cloning vector pUC18 to allow insert sequence analysis. Subsequently the Fc fragment was recloned in the baculotransfer vector pVL1393 (Pharmingen) as a BamHI-EcoRI (pUCrestriction site) DNA fragment. PVL1393 is a polyhedrin promoter controlled transfer vector designed for insertion of foreign expression cassettes in the polyhedrin locus of a baculovirus genome. The resulting transfer vector , named pVL-Fc fusion vector

(ICCG3048) (Figure 1), allowed in frame insertion of both the ED-cDNA of hB7.1 and hB7.2, using the BamHI cloning site.

#### 3.1.1.2. Cloning of full-size hB7.1 and hB7.2 cDNA sequences

5

The full size hB7.1 encoding cDNA sequence was originally generated by RT-PCR performed on total RNA isolated from EBV-transformed human spleen cells, using a hB7.1 specific primerset (MR67/MR68) designed based on the hB7.1 sequence, published by Freeman et al., 1989, as described in de Boer, M. et al., 1992. The amplified DNA was cloned in several eukaryotic expression vectors including pcDNA1neo (Invitrogen) for mammalian expression. The resulting plasmid was named pcDNA1neohB7.1 (ICCG1713).

10

The full size hB7.2 cDNA sequence was originally cloned by RT-PCR on human peripheral blood mononuclear cells using hB7.2 specific primerset IG2834/IG2833. The amplified fragment was inserted blunt end in the EcoRV opened cloning vector pBSK(+) for sequence confirmation, and later reinserted in the mammalian expression vector pcDNA3 as an EcoRV-XbaI DNA fragment, resulting in pcDNA3hB7.2 (ICCG2307).

15

#### 3.1.1.3. Insertion of the hB7.1ED and hB7.2ED in pVL-Fc

20

The cDNA sequences coding for the ED of hB7.1 and hB7.2 were then isolated by PCR on the above described plasmids pcDNA1neohB7.1 and pcDNA3hB7.2, containing respectively hB7.1 and hB7.2 full length cDNA, and using hB7.1ED or hB7.2ED-specific primersets. The resulting amplified DNA fragments were originally inserted in the cloning vector pUC18 for sequence confirmation of the inserts and later reinserted in the pVL-Fc (ICCG3048) by BamHI-BglII cloning, resulting in in frame fusion of the B7.1ED and B7.2ED to the IgGγ1-Fc cDNA sequence, already present in the pVL-Fc transfer vector, resulting in respectively the pVLshB7.1-Fc (ICCG3005) and pVLshB7.2-Fc (ICCG3004) baculotransfer plasmids (figure 2-3).

25

30

### 3.1.2. Construction of baculo transfer plasmid for hB7.1glu-glu recombinant baculovirus generation

Soluble hB7.1glu-glu was expressed as a fusion protein consisting of the extracellular domain (AA1-242) of the hB7.1 protein carboxyterminally fused to the peptide EEEEYMPME, also named glu-glu epitope, for which monoclonal antibodies are available. The hB7.1glu-glu encoding fusion cDNA sequence was generated by RT-PCR performed on total RNA isolated from an EBV-transformed human spleen cell line, using a hB7.1ED specific primerset (MR67/ MR145) based on the hB7.1 sequence published by Freeman et al., 1989, as described in de Boer, M. et al., 1992.

The sense primer matched with the codons for aminoacids 1 and 2 of the hB7.1 and contained a PstI cloning site upstream this ED-coding sequence. The antisense primer matches amino acid-codons 203-242 of the hB7.1 sequence followed by the sequence encoding the glu-glu detection/purification epitope, and a KpnI cloning site (figure 4).

#### MR67sense primer:

PstI

5' gcg ctacag catctgaagcc atg ggc c 3'

Met Gly (AA1-2 of hB7.1 preprotein)

#### MR145 antisense primer:

KpnI

5' gcgc ggtacc tta ctc cat ggg cat gta ttc ctc ttc ctg gtt atc agg aaa atg ctg ttg 3'

Glu Met Pro Met Tyr Glu Glu Glu Glu Asn Asp Pro Phe

His Gln Gln

Glu-glu epitope --hB7.1 Asn<sub>242</sub>-Gln<sub>292</sub>

The resulting 779bp PCR fragment was inserted in the baculo transfer vector pAcC8, a classical pAcYM1 derived transfer vector (Matsuura et al., 1987), as a PstI-

KpnI fragment. The resulting pAcC8hB7.1glu-glu transfer plasmid was used for insertion of the polyhedrin promoter controlled hB7.1glu-glu expression unit in the baculovirus polyhedrin locus by in vivo homologous recombination as described herunder.

5

### 3.1.3. Construction of baculo transfer plasmid for hB7.2his recombinant baculovirus generation

Soluble hB7.2his was expressed as a fusion protein of the extracellular domain (AA1-238) of B7.2 carboxyterminally fused to a six histidine peptide. The hB7.2his tagged fusion cDNA sequence was generated by PCR amplification performed on the plasmid pcDNAhB7.2(ICCG2307), containing the full size hB7.2 cDNA sequence as a template, using primer set IG8568 and IG8569. The PCR fragment, encoding AA 1<sub>Met</sub>-239<sub>His</sub> of the hB7.2 preprotein followed by 5 extra  
15 histidine residues (figure 5), was inserted in the baculovirus transfer vector pAcSG2 (Pharmingen, San Diego) as a XhoI-BglII fragment. The resulting pAcSG2hB7.2his transfer plasmid (ICCG3519) was used for insertion of a polyhedrin promoter controlled hB7.2his expression unit in the baculovirus polyhedrin locus by in vivo homologous recombination.

20

Sense primer IG8568 :

XhoI

5' ccg cicgag ccttgcaat atg gga ctg 3'

Met Gly Leu (AA 1-3 of hB7-2)

25

Antisense primer IG8569 :

BglII

3' ggg ggt ctg gtg gta gtg gtg gta gtg att cct tctaga agg 5'

Pro Pro Asp His His His His His His (AA 236-239 of hB7.2 + 5 His)

30

### 3.2. Generation recombinant baculovirions by in vivo homologous recombination



Recombinant baculovirions containing the information for the expression of the different hB7.1 or hB7.2 soluble proteins were generated following the 'baculogold transfection method' as described by Pharmingen.

5 In the baculotransfer plasmids described above pVLhB7.1Fc, pVLhB7.2Fc, pAcC8hB7.1glu-glu and pAcSG2hB7.2his, the fusion cDNA's were placed under transcriptional control of the strong late baculoviral polyhedrin promoter. The resulting expression cassette (promoter and cDNA) was flanked by baculoviral genome sequences derived from the polyhedrin genelocus, a locus non-essential for *in vitro* virus replication.

10 The recombinant transfer plasmids were introduced by classical transfection methods in insect cells (*Spodoptera frugiperda*, Sf9) together with the linearized genomic DNA of a modified version of the wild type AcNPV baculovirus genome, the Baculogold™. In this Baculogold™ baculovirus genome the polyhedrin gene is replaced by the E.coli lacZ gene and contains three Bsu36I restriction sites inside respectively the lacZ gene and the baculoviral genes ORF603 and ORF1629, originally flanking the polyhedrin locus. Bsu36I linearization of this genome results in deletion of the lacZ insert and part of the baculovirus gene ORF1629 which is essential for *in vitro* replication of the virus.

15 20 *In vivo* homologous recombination between transfer plasmid and Baculogold™ genome at the polyhedrin locus flanking sequences, resulted in the insertion of the fusionprotein expression cassette in the baculoviral genome (polyhedrin locus) and in the rescue of the lethal deletion in ORF1629. Using this approach a recombination efficiency of >99% was obtained, as wild type Baculogold™ DNA did not encode viable virus.

25 Individual recombinant baculovirions were subsequently isolated from the supernatant of transfected cells, harvested 5 days post-transfection, by a plaque-purification assay.

30 Routinely 3 candidate recombinant baculovirions were isolated for detailed protein expression pattern analysis.

### 3.3. Analysis of protein expression pattern of recombinant baculovirus infected cells.

5           The isolated recombinants were characterized by kinetic infection studies in Sf9 cells (infection at MOI3, harvest cell CM at 24, 48, 72 h post-infection) and analysis of the secreted recombinant fusion proteins by (1) Western blot using monoclonal antibodies specific for the Fc-tail (anti-Fc mAb) or specific for the glu-glu tag (anti-glu-glu mAb) or for the his-tag (anti-his mAb), and (2) in binding-  
10 inhibition studies using anti-B7.1, -and anti-B7.2 mAbs on B7.1 and B7.2 expressing cell lines.

#### Western blot analysis :

15       The *hB7.1ED* and *hB7.2ED* fusion proteins were secreted as a mature glycosylated monomer protein with M.W.  $\pm$  62kDa (12.5% SDS-Laemmli, reducing conditions , Western blot analysis with anti-Fc mAb). In non-reducing conditions the proteins showed a high degree of oligomerisation. Theoretical M.W. calculated for the non-glycosylated proteins is 53827 Da for *hB7.2ED* fusion protein and 54298 Da for  
20 *hB7.1ED* fusion protein. Optimal harvest time was determined at 72h post-infection for both proteins.

      The *hB7.1ED glu-glu* protein was secreted as a mixture of mature differentially glycosylated monomer proteins with M.W.  $\pm$  33kDa (12.5% SDS-Laemmli, reducing conditions , Western blot analysis with anti-glu-glu mAb). Theoretical  
25 M.W. calculated for the non-glycosylated protein is 28768 Da. Optimal harvest time was determined at 72h post-infection. Total deglycosylation of the protein was obtained with

N-glycosydaseF.

      The *hB7.2his* protein was secreted as a mixture of mature differentially  
30 glycosylated monomer proteins with M.W.  $\pm$  33kDa (12.5% SDS-Laemmli, reducing conditions , Western blot analysis with anti-his mAb). Theoretical M.W.

calculated for the non-glycosylated protein is 27799 Da. Optimal harvest time was determined at 72h post-infection.

Binding-inhibition assay on RPMI8866 cells:

5

Both B7.1ED and B7.2ED fusion proteins as well as the B7.1ED glu-glu and the B7.2his proteins were analysed in a binding-competition test on an EBV transformed human B-cell line RPMI8866 expressing hB7.1 and hB7.2. Dilutionseries of the crude infected cell conditioned medium (CM) was incubated with a fixed, suboptimal concentration of respectively anti-B7.1(B7-24)(20ng/cell pellet) or anti-B7.2(1G10)(30 ng/cell pellet) mAbs. The antigen/antibody mixture was subsequently incubated with the RPMI8866 cells and anti-B7.1 or anti-B7.2 antibody binding on the cells was then determined by Fluorochrome activated cell scan (FACs) using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (Fab) antibodies. Inhibition of the anti-B7 mAb binding on the cells by the B7ED fusion proteins or the B7.1EDglu-glu or B7.2EDhis proteins present in the tested sample was considered as a relative measure for the B7ED-protein concentration and functionality. Results are indicated as the highest possible sample dilution giving maximum inhibition of B7-specific mAb binding.

10

15

20

Soluble B7 fusion protein	Highest dilution resulting in maximum inhibition anti-B7.1 /anti-B7.2 binding
hB7.1Fc	1/16
hB7.2Fc	1/64
hB7.1glu-glu	1/4
hB7.2his	1/8

25

3.4. Production and purification of recombinant baculovirus expressed soluble hB7.1ED or hB7.2ED fusion proteins, B7.1ED glu-glu or B7.2ED his proteins.

30

High titre recombinant baculovirus stocks, prepared by classical virus amplification procedures, were used for production of the recombinant proteins following an in house optimized production procedure on plastic roller bottles.

5 Insect cells (e.g. Sf9) exponentially growing in spinner cultures in TC100/10% FCS medium (Gibco) were collected by low speed centrifugation, resuspended at  $2 \times 10^6$  c/ml in TC100 and seeded in glass roller bottles at a maximum volume of 600ml/R1260 cm<sup>2</sup> surface, allowing optimum aeration of the cells. A high titre virusstock was added at a multiplicity of infection (MOI) of 3. Cells and virus were

10 incubated on a roller apparatus at 27°C for 48 h. Conditioned medium (CM) was harvested and cleared from cells and cell debris by low speed centrifugation. Alternative host cell lines (e.g. Sf21, High Five), media and production procedures (e.g. spinner, bioreactor) are used.

Quality control on the CM was performed by (1) Western blot analysis, using Fc

15 specific mAb or anti-glu-glu mAb or anti-his mAb, and (2) hB7.1 and hB7.2 binding/competition assays by FACScan on hB7.1 and hB7.2 expressing recombinant cell lines, as described previous. CM was stored at -70°C until purification.

Purification *hB7-1ED* and *hB7-2ED* fusion proteins was done on a Prot A

20 column, at neutral pH and at low salt concentrations. To avoid the problem of the presence of aggregates and the formation of aggregates during the purification procedure (due to the high amount of Cys present in the whole protein (11) and due to the fact that the Cys in the Fc region has the tendency to dimerise) DTT was added in the purification procedure. DTT was added in a concentration of 2mM at

25 the starting material.

Purification of the *hB7.1gluglu*, expressed from Baculo virus infected cells, occurred by immunoaffinity onto a M24-coupled divinylsulfone activated matrix in expanded bed mode (*UpFront, Denmark*). The bound material which was eluted by pH 3.0 behaved on SDS-PAGE Western blotting (detection with anti gluglu) and

30 coomassie staining under reducing and non-reducing conditions as a monomer (MW : 35-40 kD). The sB7.1 monomeric band had a triplet nature with a smear in

between which is thought to be caused by different gradations of glycosylation. On  
gelfiltration chromatography performed with a Superdex 200 PC3.2/30 ( Smart,  
Pharmacia) (Figure 6., gelfiltration profile of  $\alpha$  : MW markers 1 = 670 kD, 2 = 158  
kD, 3 = 44 kD, 4 = 17 kD, 5 = 1.3 kD, b : sB7.1ED gluglu) , a single peak at the  
5 elution place for  $\alpha$  44 kDa protein could be detected, indicating that the  
sB7.1gluglu behaves as a monomer.

Purification of the hB7.2His6, expressed from Baculo virus infected cells,  
occurred by capturing the glycosylated proteins on Lentil lectin chromatography.  
Elution of the Lentil lectin column was performed by applying buffer containing 500  
10 mM Methylmannopyranoside. The only remaining contaminating protein with a  
Molecular weight of +/- 70 kD was removed by  $\text{Ni}^{2+}$ -IDA chromatography. The 70  
kD protein was recovered in the flow through, while the sB7.2his was recovered in  
the 200 mM imidazole elution. SDS-PAGE Coomassie staining revealed that the  
resulting material was of high purity >90%. SDS-PAGE Western blotting (detection  
15 with anti His) run under reducing conditions showed a monomeric band which  
had a triplet nature and smear in between (MW : 35-40 kD). SDS-PAGE Western  
Blotting run under non-reducing conditions showed also as major amount the  
same triplet with in between a smear but supplementary a band at the place for  
dimeric material and some higher aggregation bands but with minor intensity.  
20 The sB7.2his behaved heterogeneously on gelfiltration chromatography performed  
with a Superdex 200 PC 3.2/30 (Smart, Pharmacia), (Figure 7: gelfiltration profile of  
 $\alpha$  : MW markers 1 = 670 kD, 2 = 158 kD, 3 = 44 kD, 4 = 17 kD, 5 = 1.3 kD, b :  
sB7.2ED His6). The major peak was around 44 kD, corresponding to the  
monomeric sB7.2. Another peak, about 5 % compared to the mean peak, eluted at  
the retention time for dimeric material (+/- 90 kD). This peak was preceded by an  
25 augmentation in absorbency, which means that also higher sB7.2 oligomers were  
present. The third determined peak (also about 5% compared to the major peak)  
eluted at 2 kD.

It is clear that this sB7.2His6 protein has a higher tendency to form oligomers than  
30 the sB7.1gluglu.

3.5. *Characterization of the B7.1ED and B7.2ED fusion proteins and the B7.1ED-glu-glu and B7.2 his protein*

Purified B7.1ED and B7.2 ED fusion molecules were tested in binding inhibition studies, using anti-B7.1 mAb (B7-B7-24) and anti-B7.2 mAb (1G10), on RPMI8866 cells expressing both B7.1 and B7.2 molecules. A suboptimal concentration of anti-B7.1 mAb (B7-B7-24) (20ng/cell pellet) or of anti-B7.2 mAb (1G10) (30 ng/cell pellet) was preincubated for 20' at 4°C with different concentrations of B7.1ED or B7.2ED fusion molecule (serial dilution ranging from 1  $\mu$ g/ml to 2ng/ml). Cells ( $0.5 \times 10^6$  cells/sample) were incubated for 20' at 4°C with the B7.1ED fusion molecule/B7-24 complexes or with the B7.1ED fusion molecule/1G10 complexes or with the B7.2ED fusion molecule/B7-24 complexes or with the B7.2ED fusion molecule/1G10 complexes. JY cells ( $1 \times 10^5$  cells/sample) The results show that a low concentration of B7.1ED fusion molecule (37.5 ng/cell pellet) specific inhibits the binding of anti-B7.1 mAb (B7-24 mAb) (20ng/pellet) and not the binding of anti-B7.2 mAb (1G10) (30ng/ cell pellet) on the RPMI8866 cells. Otherwise, the results show that a low concentration of B7.2ED fusion molecule (37.5 ng/cell pellet) specific inhibits the binding of anti-B7.2 mAb (1G10) (30ng/ cell pellet) and not the binding of anti-B7.1 mAb (B7-24 mAb) (20ng/pellet) on the RPMI8866 cells.

Purified B7.1ED glu-glu molecule and unpurified B7.2ED his molecule (baculovirus supernatant) were tested in binding inhibition studies as described above, using anti-B7.1 mAb (B7-24) and anti-B7.2 mAb (1G10), on RPMI8866 cells expressing both B7.1 and B7.2 molecules. Cells ( $0.5 \times 10^6$  cells/sample) were incubated for 20' at 4°C with the B7.1ED glu-glu/B7-24 complexes or with the B7.2ED his/1G10 complexes. The experiment was further performed as described above. The results show that low concentrations of B7.1 giu-glu molecule (7.5 ng/pellet) are able to inhibit the binding of anti-B7.1 mAb (B7-24) (20ng/cell pellet) to the RPMI8866 cells and that unpurified B7.2 his (baculovirus supernatant) is able to inhibit the binding of anti-E7.2 mAb (1G10) (30 ng/cell pellet) on the RPMI cells.

Purified B7.1ED and B7.2ED fusion molecules were tested in ELISA , using anti-B7.1 mAb (B7-24) and anti-B7.2 mAb (1G10).Purified B7.1ED or purified B7.2ED fusion

molecule were coated (4°C, overnight) on Nunc Maxisorb strips in carbonate buffer, pH 9.6 at different concentrations (500-50-10-0 ng/ml). After washing 5 times with PBS, wells were subsequently blocked with PBS 0.1% casein (1 hour, 37°C) and incubated with anti-B7.1 mAb (B7-24) (concentration range, 1/2 serial dilution, 1000 to 1 ng/ml) or with anti-B7.2 mAb (1G10) (1000 to 1 ng/ml) respectively. After washing 5 times, wells were incubated (1 hour, 37°C) with HRP conjugated Goat anti-mouse IgG Fcγ specific monoclonal antibody. The ELISA was developed with the substrate TMB. Plates were measured in a ELISA reader at 450 nm. Results show that B7.1ED and B7.2ED fusion molecules can bind anti-B7.1 monoclonal antibody (B7-24) and anti-B7.2 monoclonal antibody (1G10) respectively at a coating concentration of 100 to 500 ng/ml.

Purified B7.1ED glu-glu molecule was tested in ELISA, using anti-B7.1 mAb (B7-24). Purified B7.1ED glu-glu molecule (0.5 μg/ml) was coated (4°C, overnight) on Nunc Maxisorb strips in carbonate buffer, pH 9.6. The experiment was further performed as described above. Results show that B7.1ED glu-glu molecule can bind anti-B7.1 monoclonal antibody (B7-24) at a coating concentration of 0.5 μg/ml.

#### 4. Generation of scFv's to B7.1 and B7.2

The anti-B7.1 mAb B7-24 and the anti-B7.2 mAb 1G10 are used for the generation of B7.1 and B7.2 scFv's respectively. Both the B7.1 and the B7.2 scFv's are used as positive controls in screening procedure for the generation of the B7.12 Mab. Moreover, they serve as the B7.1-, B7.2-binding component in the bispecific diabody, the bispecific BiTAB and the trispecific triabody (see further). Finally, these scFv's are used as competitor reagents in the selection for high affinity variants of the anti-B7.12 scFv.

##### 4.1. Cloning VH and VL regions by PCR in pCANTAB5E vector

Anti-B7.1 (B7-24) Mab VH and VL regions available in a baculo expression vector are previously described in WO 94/01547. Starting from this vector, both

variable regions were PCR-amplified using the degenerate primers available from the Pharmacia RPAS (Recombinant Phage Antibody System) Mouse scFv Module. In a second PCR, both VH and VL were linked using a short synthetic linker. After cleavage with the SfiI and NotI restriction sites, de B7-24 scFv was ligated in  
5 pCANTAB5E. To select for functional scFv's, phages were generated after ligation in pCANTAB5E. TG1 cells were transformed with the ligation mixture and were infected with M13K07 helper phage to induce phage production. The produced phages were panned upon a B7.1 positive B cell line (JY cells), washed and subsequently eluted from the JY cells. Binding phages were screened for their  
10 binding capacity in a FACS analysis with this B7.1 positive B cell line. Correct binders were selected for further DNA sequence analysis. The sequence of B7-24 scFv clone 6 was found to be correct.

For the anti-B7.2 scFv's, the VH and VL regions were cloned by RT-PCR with a set of degenerate forward primers located at the 5' end of the VH or VL region and a  
15 backward primer located in the constant region of the heavy or light chain. The resulting cDNA was cloned in pUC18 and consensus VH and VL sequences were determined.

Starting from correct clones in pUC18, the VL and VH regions were cloned in the pCANTAB5E vector using degenerated primers obtained from Pharmacia,  
20 transformed in TG1 cells, infected with M13K07 helper phage, followed by panning upon a B7.2 positive B cell line (JY cells), as described for the anti-B7.1 regions. Correct binders were selected for further DNA sequence analysis. The sequence of 1G10scFv clone 31 was found to be correct.

#### 25 4.2. Test expression of scFv's with phage FACS and ELISA

The recombinant phages expressing B7-24 scFv( clone 6) and 1G10 scFv (clone 21) were tested in FACS experiments using JY cells, expressing B7.1 and B7.2 molecules (de Boer et al., 1992). JY cells ( $1 \times 10^5$  cells/sample) were incubated for 20'  
30 at 4°C with B7-24 scFv or 1G10 scFv phage diluted serial from undiluted to zero. After washing twice in RPMI1640 supplemented with 10% FCS, the cells were



subsequently incubated for another 20' at 4°C with a sheep anti-M13 monoclonal antibody, washed twice in RPMI1640 supplemented with 10% FCS and incubated (20' at 4°C) with anti-sheep antibody conjugated to fluorescein isothiocyanate (FITC). JY cells were washed twice in RPMI1640 supplemented with 10% FCS and finally suspended in PBS supplemented with 1% BSA and 0.1 % NaN<sub>3</sub> and analysed in a FAC-Scan flow cytometer (Becton Dickinson). The specific binding of the scFv phages is expressed as the mean fluorescence intensity in arbitrary units. The results showed that the B7-24 scFv and the 1G10scFv phages binds to the JY cells. The mean fluorescence intensity (MFI) diminished when the phage is titrated.

Evidence showing that the obtained scFv phages are specific for B7.1 or B7.2 was obtained in a competition FACS experiment on JY cells, expressing B7.1 and B7.2 molecules. In this competition experiment the JY cells were preincubated with the anti-B7.1 mAb (B7-24) or the anti-B7.2 mAb (1G10), followed by incubation with the scFv phages. JY cells (1x 10<sup>5</sup> cells/sample) were incubated for 20' at 4°C with 2µg anti-B7.1 mAb (B7-24) or with 2µg anti-B7.2 mAb (1G10). After washing twice in RPMI1640 supplemented with 10% FCS, the cells were incubated for 20' at 4°C with B7-24 scFv or 1G10 scFv phage diluted serial from undiluted to zero. After washing twice in RPMI1640 supplemented with 10% FCS, the cells were subsequently incubated for another 20' at 4°C with a sheep anti-M13 monoclonal antibody, washed twice in RPMI1640 supplemented with 10% FCS and incubated for 20' at 4°C with anti-sheep antibody conjugated to fluorescein isothiocyanate (FITC). JY cells were washed twice in RPMI1640 supplemented with 10% FCS and finally suspended in PBS supplemented with 1% BSA and 0.1 % NaN<sub>3</sub> and analysed in a FAC-Scan flow cytometer (Becton Dickinson). The specific binding of the scFv phages is expressed as the mean fluorescence intensity in arbitrary units. The results showed that the B7-24 scFv phage is not capable to bind the JY cells preincubated with the parent monoclonal antibody, B7-24. No inhibition of the binding of the B7-24 scFV could be detected when JY cells were preincubated with anti-B7.2 monoclonal antibody, 1G10. Similar results were obtained for the 1G10 scFv phage. The 1G10 scFv phage is not capable to bind the JY cells preincubated with the parent monoclonal antibody, 1G10. No inhibition of the binding of the 1G10

scFv could be detected when JY cells were preincubated with anti-B7.1 monoclonal antibody, B7-24.

Functional binding of the recombinant phages to their respective antigens was also tested in ELISA using the B7.1ED and B7.2ED fusion proteins. Plates were coated overnight at 4°C with anti-human IgG. After washing with PBS Tween, plates were incubated with B7.1ED or B7.2ED fusion proteins. After washing (PBS Tween), the plates were incubated with the phages (B7-24 phage or 1G10 phage) in serial dilution from undiluted to zero or with the parent monoclonal antibodies (B7-24 or 1G10) serially diluted from 100ng/well to zero. Binding was detected with a HRP labelled anti-M13 or anti-mouse Ig antibody. As substrate TMB was used. Plates were measured at 450 nm. All incubations were done for 1 hour at 37°C.

Results showed that B7-24 scFv and 1G10 scFv bind to the B7.1ED or B7.2ED fusion molecule respectively. The binding of the scFv phages is much higher than the binding of the parent antibodies. This is not due to a difference in binding affinity, but is the result of the different detection systems used.

In case antibody VH and VL regions do not directly fold properly as scFv's, a small PM library in the pCantab5E vector (see section 6) is generated and specific mutations in VH-VL pairing domains are introduced in order to optimize scFv folding. Screening for variants with improved folding is done using immobilized ED fusion proteins.

#### 4.3. Milligram scale production and purification of scFv's in *E. Coli*

To produce milligram amounts of purified ScFv, cDNA encoding the scFv's from the pCantab5E vector are transferred to an *E. coli* expression vector. The genes encoding the scFvB7-24H6 and scFv1G10H6 were isolated by PCR with primer 6999 and 7002 for scFvB7-24 and primer 7001 and 7002 for scFv1G10, whereby specific restriction sites were created.

list of used primers:

6999: 5'-CGCGGTATACAGGAGTCTGGGGGAGGCTTAG-3'

7000: 5' -CGCGCTCGAGCTTGGTCCCCTGACCGAAC- 3'

7001: 5' -CGCGGTATACAGCAGTCTGGACCTGAGCTG- 3'

7002: 5' -CGCGAGCGCTTGGTACCTCCACCGAACG- 3'

5 The PCR fragments were cloned in the pGEM-T-vector (Promega) and were subjected to DNA sequence analysis. After cleavage with *XhoI* - *Bst1107* and *Eco47III* - *Bst1107*(Biolabs) of pGEM-TscFvB7-24H6 and pGEM-TscFv1G10H6 respectively, the genes encoding scFvB7-24H6 and scFv1G10H6 were cloned in an *E.coli* expressionvector under control of the IPTG inducible lac-promotor. They are preceded by the pelB signal sequence to obtain secretion into the periplasm. A C-terminal histidine-tag for purification purposes was included. The expression plasmids containing the scFv constructs were transformed in the *E.coli* expressionstrain JM83. (Knappik and Plückthun 1995). An overnight culture was 20x diluted in LB+ 100µg/ml amp+ 1% glucose and incubated at 28°C until an OD600 of 0.5 was reached. After removing the glucose of the medium, the culture was 15 induced with 0.1mM IPTG and further incubated at 28°C during ±18h. To isolate soluble periplasmic proteins, the method described by Neu and Heppel (1965) was used. Briefly, cells were harvested by centrifugation and resuspended in ice cold shockbuffer (100mM Tris-HCl pH 7.4; 20% sucrose, 1mM EDTA, pH8). After 20 incubation on ice during 10' with occasional stirring, the mixture was centrifuged at 10.000rpm during 1,5'. The supernatans was removed and the pellet was immediately resuspended in ice cold distilled water. After incubation on ice during 10' with occasional stirring, the mixture was centrifuged at 14.000rpm and the obtained supernatans was the soluble periplasmic fraction. Only 10-30 % of the 25 expressed scFv 's was found in the periplasm as a soluble and correctly folded functional molecule and a small amount leaked in the culture supernatans. This small periplasmic fraction was purified using metal affinity chromatography. ScFv B7-24 from the soluble periplasmic fraction was purified up to >90% purity by applying it onto a Zn<sup>2+</sup>-IDA column with subsequent wash and elution with 40 and 30 150 mM Imidazole. The same procedure is applied for the purification of ScFv 1G10 from the soluble periplasmic fraction..

The major part of the scFv's formed inclusion bodies in the periplasm but were processed correctly. This was confirmed by NH<sub>2</sub>-terminal sequence analysis.

#### 4.4. Characterization and BIAcore analysis of scFv's

Binding specificity of the expressed scFv's is checked by ELISA experiments using the ED fusion proteins, and FACS analysis using EBV-transformed cells (RPMI 8866), that are positive for expression of both B7.1 and B7.2 molecules. Binding of scFv's to their target molecules is detected by means of a his-tag present on these scFv's.

In the ELISA experiment, mouse anti-his mAb (1/1000 conc) is coated on the plate in PBS for 2h at 37°C. After washing, B7-24 scFv is added in a twofold serial dilution starting from 1 µg/ml to 15 ng/ml and incubated for 1h at 37°C. The full size mAb B7-24 was added as positive control (1 µg/ml). After washing, the B7.1 ED fusion protein (500ng/ml) is added for 30' at 37 °C and detected by sheep anti-human HRP (conc, 1 hour, 37 °C). TMB was added as substrate. Results were measured at 450nm. Results showed that purified B7-24 scFv (2 µg/ml) binds the B7.1 ED fusion protein in this ELISA.

Purified B7-24 scFv was also tested and compared with the full size mAb B7-24 for its binding capacity to the human B7.1 molecule present on the RPMI8866 cells, a EBV transformed B cell line positive for hB7.1 and hB7.2. RPMI 8866 cells were incubated with B7-24 scFv or, as control, with anti-B7.1 mAb (B7-24). Cells (0.5 x10<sup>6</sup>/sample) were incubated for 30' at 4°C with B7-24scFv or with anti-B7.1 mAb (B7-24). After washing twice in RPMI supplemented with 10% FCS, the cells were incubated for another 30' at 4°C with a mouse anti-His mAb. After washing twice in RPMI supplemented with 10% FCS, the cells were incubated for another 30' at 4°C with a biotinylated goat anti-mouse IgG(Fab)-antibody followed by an incubation (30' at 4°C) with streptavidine conjugated with phycoerythrine (PE). The cells were washed twice in RPMI 1640 supplemented with 10%FCS and finally suspended in PBS supplemented with 1% BSA and 0.1 % NaN<sub>3</sub> and analyzed with a FACS Scan flow cytometer (Becton Dickinson). The specific binding of the monoclonal

antibodies is expressed as the mean fluorescence intensity in arbitrary units. The results show that the B7-24scFv binds to the RPMI 8866 cells, expressing both B7.1 and B7.2 molecules.

The purified scFvB7-24 were tested for their capacity to block the T cell APC interaction in a mixed lymphocyte culture. T cells were purified out of whole heparinized blood on Ficoll-Paque (density 1.077, Pharmacia Biotech) density gradients. The peripheral blood mononuclear cells (PBMC) present in the interface were washed three times in 40 ml of RPMI supplemented with 10% inactivated FCS. Subsequently, monocytes were removed by cold aggregation (Mentzer et al., 1986). To this end, PBMC were resuspended in 40 ml of RPMI supplemented with 10% inactivated FCS and slowly rotated for 30' at 4°C. Monocyte aggregates were allowed to sediment over a 15' period incubation on ice, and the non-aggregated cells containing enriched T cells and B cells were carefully aspirated and centrifuged for 10' at 1200 rpm. T cells were further enriched using Lympho-Kwik-T (One lambda Inc, Los Angeles, CA). This reagent contains a mixture of anti-monocyte and anti-B cell mAbs and complement. Lymphocytes were resuspended in 3 ml Lympho-Kwik-T and the mixture was incubated for 45' at 37°C. Subsequently, cells were resuspended in 0.5 ml of PBS and centrifuged for 2' at 2000 rpm and washed twice. The mouse fibroblast cell line, 3T6, transfected with the cDNA encoding human B7.1 and human CD32 (3T6/CD32/B7.1) was mitomycin C treated. Cell pellet of 1 subconfluent falcon was dissolved in 800 ml RPMI and 200 ml Mitomycin C (250 mg/ml) and incubated for 40' at 37°C followed by two washes. Subsequently, cells are suspended in 30 ml RPMI and incubated for 15' at 37°C followed by one additional wash step. In the mixed lymphocyte culture (MLR), the mitomycin C treated 3T6/CD32/B7.1 cells ( $10^4$  cells/well) were incubated with OKT3 (1  $\mu$ g/ml) for 1 h at 37°C followed by a 1 h incubation at 37°C with decreasing concentrations of mAbB7-24 or scFvB7.24. Subsequently, purified T cells ( $5 \times 10^4$  cell/well) were added and incubated for 5 days. After 5 days, cells were incubated with 1 mCi ( $^3$ H)-thymidine for 6 h and harvested using an automated cell harvester. ( $^3$ H)-thymidine incorporation was determined with a liquid scintillation counter.

Results showed that the recombinant scFvB7-24 antibodies displayed neutralizing activities, comparable to those of the parent B7-24 mAb, as measured in MLR.

The binding affinity of the scFv's is compared with that of Fab fragments of the parent antibody in order to evaluate whether the scFv fragments have the same affinity as the intrinsic affinity of the parent antibody, which is reflected in the binding characteristics of the Fab fragments. The ED fusion proteins are used as target molecules and the conditions for appropriate analysis of the different molecules are optimized for BIAcore analyses.

These accumulated data give an indication of the extend with which the affinity is lowered by the construction of a scFv and set a goal for the increase in affinity obtained using Parsimonius mutagenesis (PM).

#### 6. Generation of novel B7.12 Mabs or small antigen binding peptides/ fragments (microproteins)

This novel molecule is a single mAb that can bind to both B7.1 and B7.2. In spite of the fact that both B7.1-B7.2 ligands, CD28 and CTLA4, bind B7.1 and B7.2 with comparable affinities, it has not been possible to produce murine Mabs which react with both B7.1 and B7.2. This is probably due to the fact that the active B7.1-B7.2 epitopes, which are conserved between B7.1 and B7.2, are also sufficiently conserved between mouse and human to ensure that idiotypes against the human epitopes would be suppressed in the mouse as anti-self.

Mice are immunized with peptides derived from the homologous sites between B7.1 and B7.2 and, as an alternative route, human Mabs which react with high affinity to both human B7 molecules are made via antibody engineering. Two types of phage display libraries are used to obtain such antibodies by biopanning on immobilized B7.1ED and B7.2ED fusion proteins. As an alternative approach, a

phage-display conformational constrained library is screened which ends up in the development of small antigen binding peptides or fragments (also called microproteins) interacting with B7.1 and B7.2.

#### 5 4.1. Phage display libraries

##### 4.1.1. Generating 'rationally' and 'random' designed CTLA4-like libraries

10 In the first series of experiments ('rational' approach), CTLA4 elements are grafted onto a limited set of structurally homologous antibody scaffolds to define which elements within CTLA4 may be replaced without losing B7.1-B7.2 binding. Otherwise, immunoglobulin sequences are grafted onto structurally and functionally important CTLA-4 sequences. CTLA4 is a member of the immunoglobulin superfamily, and as such its main extracellular region is built up of  
15 a domain with a basic immunoglobulin fold. On the basis of a model of CTLA4 and a comparative analysis to known antibody variable domains, it is determined which CTLA4-elements are grafted (CDR1-3 including the 'MYPPPY' sequence, possibly FR residues), onto which scaffold. The 'scaffolds' are derived from the structurally most homologous antibody V-genes. For example, FR2 and FR3 are most  
20 homologous to human lambda light chains with respect to amino acid sequence, with some homology for human kappa regions. 'Structural' homology, which in our case is the similarity between antibody structure and the CTLA-model, aided by predictions on similarity of CDR-canonical folds determine which 'scaffolds' are used. This part of the work requires detailed computer modelling and is performed  
25 by Dr. Johan Desmet, K.U.Leuven, Campus Kortrijk, Interdisciplinair Research Centrum, Belgium (Desmet et al., 1992; Lasters and Desmet, 1993; Lasters et al., 1995 and Desmet et al., 1997).  
Such chimaeric molecules are created and tested for binding to B7.1 and B7.2; this generates very useful information for the second stage of this approach. Large  
30 repertoires of selected antibodies (and their FR) are then made and are provided with a minimum of CTLA4-elements; these molecules are displayed on the surface

of phage. From these hybrid libraries molecules that bind to both B7.1 and B7.2 are selected.

The CTLA4 CDR regions are shuffled at the DNA level with the selected antibody FR sequences; oligonucleotides encoding the CTLA4 CDR regions fused to  
5 neighbouring antibody-derived framework residues are combined with 4  
repertoires of frameworks (FR1-4) by PCR-assembly. Certain templates (VH, V-  
kappa or V-lambda) are chosen based on successful identification of closely  
homologous FR sequences.

10 The display of CTLA-4 on filamentous phage is a pre-requisite for the rational  
design approach using CTLA-4 as a scaffold. We have designed primers to clone  
human CTLA-4 into our phage display vector pCES1. We have cloned hCTLA-4  
extracellular domain into pCES1 as a *Apa*1/*Not*1 fragment. This fuses CTLA-4 to  
the N-terminus of p3 for display on filamentous phage. We have decided to use a  
15 CTLA-4 fragment as described in (Metzler *et al.*, 1997) except we will not include  
the C-terminal cysteine residue (Cys 123) believing that this may present problems  
with the correct folding and presentation of the CTLA-4 p3 fusion on filamentous  
phage. In figure 9 the PhagemidpCES1 is represented : antibody genes :  $V_L-C_L$ ,  
variable (V) and constant (C) region of the light chain;  $V_H-C_{H1}$ , variable and first  
20 constant region of the heavy chain; *PlacZ*, promoter; rbs, ribosome binding site; S,  
signal sequence; H6, six histidines stretch for IMAC purification; tag, c-myc-derived  
tag; amber, amber codon that allows production of soluble Fab fragments in non-  
suppressor strains, gIII, gene encoding one of the minor coat proteins of  
filamentous phage. Restriction sites used for cloning are indicated.

25 Clones with the correct sized insert were further tested by *Bst*NI fingerprint and  
shown to have the expected pattern. Phage were prepared and tested in ELISA for  
binding to B7-1Ig and B7-2Ig. A strong signal was obtained showing binding to both  
B7.1-Ig and B7.2-Ig. and not to BSA or plastic . Soluble CTLA-4 also showed some  
binding to B7-1 and B7-2 in ELISA, however the signal was only twice background.

30 It is interesting to see what the enrichment factor is of CTLA-4 phage for the  
ligands B7-1 and B7-2, and to determine if it will indeed be possible to select novel



binding molecules. CTLA-4 binds to B7-1 and B7-2 with  $K_d$  of 0.4 and 2.2  $\mu$ M respectively with a very fast off rate ( $k_{off} > 0.4 \text{ sec}^{-1}$ ) (Greene et al., 1996; Van der Merve et al., 1997). The enrichment factor of CTLA-4 phage on B7-1Ig and B7-2Ig was 5100 and 2687 respectively. This enrichment factor is very high, of the order  
5  $10^3$  per round of selection and is presumably due to the avidity effect of display on filamentous phage. This means that, provided chimeric CTLA-4/Vgene molecules are expressed and displayed efficiently, that selection is possible even for lower affinity interactions than the wild-type CTLA-4/B7-1 interaction.

In a second rational design approach using immunoglobulin as a scaffold, a  
10 rational protein design can identify a structurally homologous protein scaffold (ie a member of the immunoglobulin superfamily (e.g  $V_H$ ,  $V_L$ , CD2, CD122, CD28) upon which we can graft structurally and functionally relevant regions of CTLA-4. For example, the immunoglobulin light chain A27(DPK22) is the most frequently used human light chain *in vivo* and can be used as a scaffold. Light chains are  
15 generally more soluble than heavy chains, and light chains are more structurally compatible with CTLA-4 than heavy chains. We have designed a molecule that can be synthesised from oligonucleotides for display on filamentous phage.

Features which have been incorporated are (Figure 10: Hybrid light chain/CTLA-4 molecule):

20 -CDR1 of CTLA-4 introduced at a structurally compatible site of A27 as EY-CDR1-VRVTV

-CDR3 of CTLA-4 introduced at a structurally compatible site of A27 as KVEL-CDR3-GIG

We have introduced degeneracy at Ileu51 and phe 65 so as to generate constructs  
25 with both the naturally occurring light chain residues at these sites and also to allow for the introduction of the non-Ig disulphide bridge which is present in CTLA-4

We have introduced degeneracy at Phe 74 so as to generate constructs with both the naturally occurring residue and also the valine (Strand E) of CTLA-4 which may be structurally important.

30 Restriction sites have been introduced by silent mutagenesis so as to be able to

replace CDR1, 2 and 3 and also further engineering of molecule based on structural, functional and solubility modelling predictions.

Restriction sites of *Apa*L1/*Not*I have been introduced for cloning into phage display vector for binding studies and further manipulation using either rational or random approaches.

The C $\kappa$  light chain is appended to the C terminus as a *Kpn*I/*Not*I fragment to solve the solubility problem, as expected with a unpaired light chain molecule.

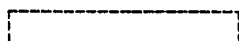
The generated molecules are manipulated in a rational way using modelling predictions to engineer changes to resolve any functional, structural or solubility difficulties. Furthermore this molecule is fed into the random approach.

In the random approach, 'random' framework pools, derived from naive or immune Ig pools are used to identify apparent compatible antibody-derived FR regions.

We have used DNA shuffling (Stemmer., 1994) to generate libraries of chimeric CTLA-4 immunoglobulin molecules. By shuffling human CTLA-4 with a pool of human V<sub>H</sub> and V<sub>L</sub> antibody chains and their subsequent display we select novel B7-1/2 binding molecules. Molecules are fused to the N-terminus of the minor phage coat protein p3 and selected for binding to B7.1/2. Our experimental strategy is shown in figure 11

We have generated two repertoires of chimeric molecules which have reamplified with primers specific for the N terminus of V<sub>L</sub> and also the C-terminal of CTLA-4. The rationale for using a C-terminal primer specific for CTLA-4 was that this is functionally the most important region and should be retained if possible in any new B7-1/2 binding molecule. The two repertoires that have been constructed in pCES1 have been DNA shuffled at 50°C and also at a lower temperature 45°C.

*Hu-CTLA-4/VL hybrid*



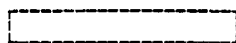
Hu-V $\kappa$  Back ApaL1 (17 primers)

Hu-CTLA-4-For-Not1 (1 primer)

<u>Type of library</u>	<u>Temperature of shuffle</u>	<u>Library size</u>
pCES1 (VL/CTLA-4)-1	45°C	1.1e6
PCES1 (VL/CTLA-4)-2	50°C	8.4 e5

We have also generated two repertoires of chimeric CTLA-4/immunoglobulin molecules displayed on phage where we have amplified with primers specific for both the N and C-termini of immunoglobulin light chains.

10

*Hu-CTLA-4/VL hybrid*

15

Hu-V $\kappa$  Back ApaL1 (6 primers)Hu-J $\kappa$ -For-Not1 (5 primers)

<u>Type of Library</u>	<u>Temperature of shuffle</u>	<u>Library size</u>
pCES1(VL/CTLA-4)-3	45°C	1.3 e 6
PCES1(VL/CTLA-4)-4	50°C	7.8 e 5

20

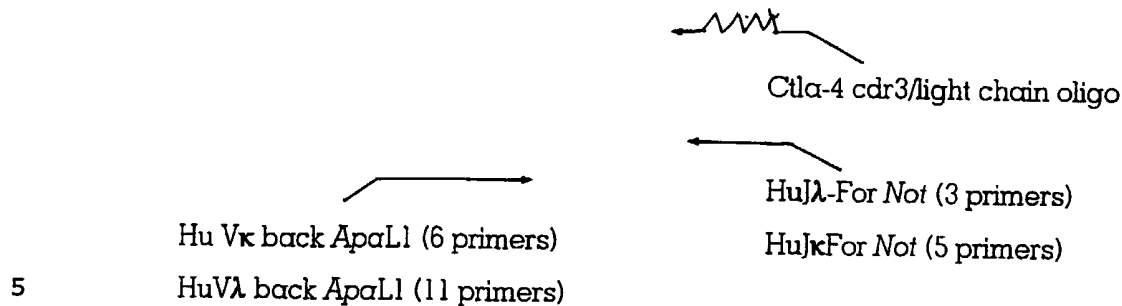
To introduce CTLA-4 CDR3 into light chain repertoire we have designed primers which can be used to introduce the functionally important CTLA-4 CDR3 sequence into a immunoglobulin light chain repertoire at a structurally compatible position. These oligonucleotides are also used to spike DNA shuffling experiments.

25

CTLA-4 CDR3/light chain oligonucleotide design: V<sub>L</sub>-FR3---MYPPPY---V<sub>L</sub>FR4

30

*Immunoglobulin light chain repertoire*



Sequences of the CTLA-4 CDR3/V $\kappa$  light chain spiking oligonucleotides are designed so as to amplify all V $\kappa$  sequences and are presented in figure 12

Sequences of CTLA-4 CDR3/V $\lambda$  light chain spiking oligonucleotides are designed so as to amplify all V $\lambda$  sequences and are presented in figure 13

In all cases it is crucial to improve the selectivity of the selected molecules for B7.1, B7.2 or both. Chain shuffling is used for this purpose.

#### 4.1.2 Generating dual-binding Fab fragments by alternating selections from a large naive antibody repertoire

This method selects antibodies binding to both B7.1 and B7.2 from a large naive Fab library, which contains  $4.1 \times 10^{16}$  antibody molecules (Target Quest) displayed on phage. Selections are alternated between (biotinylated) B7.1ED and B7.2ED fusion protein and with excess of non-labeled Ig to deplete Ig-binding antibodies. This selection mode yields antibodies that target the (partially) overlapping binding site of CTLA4 on B7.1 and B7.2. It is also necessary to ensure the removal of antibodies specific for the junction between B7.1/2 and the Fc moiety; therefore alternating selections are done with hB7.1glu-glu and B7.2 his proteins, without the Fc portion. Gene diversification is required to improve the selectivity of the antibody. For this, the library quality is optimized using Trinucleotide-directed Mutagenesis (TRIM) technology from Morphosys (Virnekäs et al. 1994).

#### 4.1.3 Generating of small B7.1 and B7.2 binding peptides/ fragments

(microproteins)

This method selects small antigen-binding peptides from random DNA sequence phage display libraries composed of cyclic peptides (Tn6, Tn8, Tn9, Tn10 and HTS) and variants from human-origin proteins (Kunitz and endothelin libraries) (described in US patent numbers 5,403,484 and 5,571,698 and 5,223,409 to Ladner et al.). These libraries allow to examine several hundred million compounds and useful 'hits' emerge. Both the structured peptide 'hits' and the human origin 'hits' give rise to suitable clinical leads. To obtain ligands that bind both B7.1 and B7.2 the libraries are screened in three different ways:

- 1) Bind to B7.1 and elution of bound phage with soluble B7.2
- 2) Bind to B7.2 and elution of bound phage with soluble B7.1
- 3) Alternate binding to B7.1 (e.g. rounds 1 and 3) and B7.2 (e.g. rounds 2 and 4)

#### 4.2. Classical immunization

In spite of the fact that we did not succeed in generating murine mAbs, reacting with both B7.1 and B7.2, upon immunization with B7.1 or B7.2 respectively, we generate crossreacting murine mAbs by immunizing homologous sites from B7.1 and B7.2. The sequences of human B7.1ED and B7.2ED were compared by an alignment. Five regions from human B7.1ED and humanB7.2ED showed homology and peptides of these sites were synthesized.

Three peptides were derived from human B7.1:

B7peptide1: region 66-94 human B7.1

B7peptide2: region 137-148 human B7.1

B7peptide4: region 200-212 human B7.1

Two peptides were derived from human B7.2:

B7peptide3: region 124-135 human B7.2

B7peptide5: region 183-194 human B7.2

The peptides were synthesized on Tentagel S resin (Rapp Polymere GmbH, Germany) using a Rainin Symphony/Multiplex synthesizer with standard Fmoc-chemistry and HOBt/TBTU (TBTU:2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBt : N-Hydroxybenzotriazole) *in situ* activation. Standard double couplings were performed using a 4 fold excess of amino acids in the presence of equimolar amounts of HOBt and TBTU for 2 x 20 minutes. The Fmoc protecting group was removed by mild base treatment using 2% piperidine/2% DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene) in DMF (Dimethylformamide).

Biotinylation was performed by dissolving biotin in 30% dimethylsulfoxide / 70% dimethylformamide with *in situ* activation. After completion of the peptide-resin complex the peptide is cleaved of the resin by incubating for 2,5 h with 90% trifluoroacetic acid/ 5% thioanisole/3% ethanedithiol/2% anisole. The peptide is precipitated from the cleaving mixture using t-butylmethylether. After centrifugation the pellet is washed 3 times with t-butylmethylether and dried overnight under vacuum. The purity of the crude peptide is checked on RP-HPLC.

The following B7 peptides were synthesized:

B7peptide1 (IGP1458): Acetyl WQKEKKMVLTTGGXGGGEYKNRTIFD - CONH<sub>2</sub>  
 B7peptide2 (IGP1459): Acetyl LSVKADFPTPSI - CONH<sub>2</sub>  
 B7peptide3 (IGP1460): Acetyl LSVLANFSQPEI - CONH<sub>2</sub>  
 B7peptide4 (IGP1461): Acetyl SQDPETELYAVS - CONH<sub>2</sub>  
 B7peptide5 (IGP1462): Acetyl SQDNVTELYDVS - CONH<sub>2</sub>

B7Peptide1, B7peptide2, B7peptide3 and B7peptide4 are biotinilated peptides.

B7peptide1 was synthesized as  $\alpha$ -branched peptide.

These peptides are able to induce an immune response in the mice.

For this purpose, mice are immunized with these different peptides, containing

overlapping sites from B7.1 and B7.2. A classical immunization scheme of 4 injections at 4 weeks interval is followed. Different well known routes of injection,

different carriers and different adjuvants are tested. Bleedings are obtained at day 0 (pre immun serum), day 66 (bleeding 1) and at day 94 (bleeding 2). Pre and post immun sera are tested in ELISA for reactivity with B7.1 and B7.2 using the B7.1ED and B7.2ED fusion proteins. Mice developing antibodies reacting with both B7.1 and B7.2 are boosted and sacrificed 3 days after the boost.

The fusion is performed using a standard protocol. Briefly, splenic cells from the immune mouse are fused with SP2/0 myeloma cells in ratio 10:3 using PEG/DMSO. Fused cells are plated in 96-well plates at density of  $5 \times 10^3$ - $10^4$  cells per well and the first screening is performed 7 to 10 days later by ELISA for reactivity with B7.1 and B7.2. Positive clones are subcloned by limiting dilution and tested for reactivity with B7.1 and B7.2. Those hybridomas reacting with both B7.1 and B7.2 are selected for further characterization.

The selected hybridomas are then tested for their affinity for B7.1 and B7.2 in BIAcore experiments. They are also tested in an *in vitro* system of human T cell-APC interaction, in order to show their efficiency as blocking agents for allogeneic T cell activation, ability to induce anergy, and ability to exclude any direct stimulating effect on the APC. The efficacy of these mAbs as immunosuppressive agents is tested in *in vitro* models as outlined further.

#### 5. Affinity maturation of anti-B7.12, anti-B7.1 and anti-B7.2 by Parsimonious Mutagenesis

The generation of humanized versions of rodent Mabs has successfully been achieved by various methods. Humanized Mabs exhibit the same ligand-binding properties as the original rodent Mabs, but in general with severely decreased binding affinity. In addition, human Mabs (huMabs) derived from naive human libraries only have primary response-level affinities, i.e., sub-micromolar. However, such affinities are still 2-3 orders of magnitude below what is usually required for therapeutic efficacy, and affinity maturation will have to be completed *in vitro*.

The affinity of the newly generated anti-B7.12 mAbs is matured using *in vitro* mutagenesis techniques. Also the anti-B7.1 and anti-B7.2 scFv, necessary for the different constructs (see further) are affinity matured.

*In vivo* antibodies are affinity matured in a stepwise fashion, by gradually

5 incorporating mutations that cause small incremental improvement of the affinity (for review see Hoogenboom H., 1997). Mutations affect affinity indirectly by influencing the positioning of side chains contacting the antigen, by providing new contact residues (particularly when they are located in or near the centre of the antigen combining site), or by replacing 'repulsive' or low affinity contact residues  
10 with contact residues with more favourable energetics. The *in vitro* affinity maturation process essentially involves three steps, (1) the introduction of diversity in the antibody V-genes, creating a 'secondary' library, (2) the selection of the higher affinity from the low affinity variants, and (3) the screening to allow discrimination of antibody variants with differences in affinity or kinetics of binding.

15 The selection is chosen to favour kinetic parameters such as off-rate or affinity; this hinges on the use of limited and decreasing amounts of antigen, and on performing the selections in solution rather than by avidity-prone panning. For example antibodies of the highest affinity can be preferentially selected by using the antigen concentration at or below the desired dissociation constant. The areas  
20 that are best targeted for mutagenesis differ for each individual antibody. Diversity is introduced either more-or-less randomly, as with error-prone PCR, by using mutator strains, by V-gene chain shuffling, or by DNA shuffling. Chain shuffling is used successfully to obtain a 300-fold increase in the affinity of an anti-hapten antibody, originally isolated from a naive human scFv library. In this experiment  
25 first the light chain, then the heavy chain segment (without CDR3) is replaced by a repertoire of human partner domains somatically mutated *in vivo*, and from these repertoires the best variants are selected on antigen. A similar approach is applied to antibodies binding to protein antigens, with a more modest 6-fold affinity improvement for an anti-c-erbB2 antibody. Chain shuffling appears to be the  
30 method of choice when the starting dissociation constant is  $> 10$  nM or the  $k_{off}$  is faster than  $10^{-3}$  sec $^{-1}$ , but is still useful even for high affinity antibodies to identify



those residues in the antibody involved in contacting the antigen, which could in a second step be (partially) randomised. For higher affinity antibodies ( $K_d < 10$  nM or  $k_{off} < 10^{-3}$  sec<sup>-1</sup>) the antibody CDR regions are targeted, using oligonucleotide directed mutagenesis or spiked oligonucleotides and PCR. With over 100 CDR residues constituting the antigen combining site, a choice is made as to where to start. If the structure of the antibody-antigen complex is known, residues that contact the antigen or may influence other residues contacting the antigen are targeted. Such residues are also determined experimentally, by chain shuffling, by alanine-scanning of the CDR-regions, by parsimonious mutagenesis (see below), or by modelling. Residues involved in maintaining the main chain conformation of the CDR are conserved; residues that modulate affinity are randomized, ideally 4-6 residues at a time to allow efficient sampling of the sequence space. Sequential targeting is preferred, as additive effects of mutants obtained by targeting CDRs in parallel and combining mutations prove frequently unpredictable. For the different antibodies that are considered for affinity maturation, the following schedules are followed.

In the CTLA-4 element containing antibodies the shuffle between CTLA-4 and antibody elements yields Fv-like molecules with an Ig-fold that bind to both B7.1 and B7.2. The affinity of these molecules is low, around 10 to 100 nM for the  $K_d$ . The maturation concerns antibody-like molecules, in which it remains unclear which areas of the molecule are involved in antigen binding. Therefore the first V-gene segment is exchanged via DNA-shuffling (basically similar to the first experiments, now with more defined subsets of V-gene derived segments), and in a parallel approach, the XL1-Red mutator strain. These methods guarantee the highest possible affinity gain without needing the 'educated guess' that is required to apply oligo-directed mutagenesis or Parsimonious Mutagenesis (see below). These experiments also indicate which CDR- and FR-residues are involved in binding, information which aid in the construction of libraries using spiked oligonucleotides and P.M. and re-selection of the repertoire.

The maturation of the human B7.1/2 antibodies concerns genuine antibodies, with dual binding activity, which facilitate choice of mutagenesis method. The method

that is applied here depends on the affinity of the starting antibody. If the off-rate is higher than  $10^{-3} \text{ s}^{-1}$ , the choice will be on chain shuffling, because this is a fast and straight forward procedure, with the highest chance to retrieve antibodies with improvements beyond this threshold. If the off-rate is slower, or has been made slower using this procedure, in the next step parsimonious mutagenesis is applied. Both the heavy and light chain CDR's are targeted, in a sequential way, to derive antibody variants, which are then selected using limited amounts of biotinylated antigens (retaining parallel dual selections to retain affinity for both target. Initially both H3 and L3 are targeted; however, other CDR's also have to be diversified, because the binding site is extraordinary broad due to the dual binding capacity. The P.M. procedure is explained further in the text.

It is necessary to affinity mature the murine B7.1 and B7.2 antibodies beyond the values that are normally found in antibodies, made by the natural immune system, as these antibodies are used to build larger molecules with two adjacent binding sites based on the murine Fv fragments.

The method of choice depends on the starting affinity, and follows the previous schedule (chain shuffle mutator strain) and as an alternative making use of spiked oligonucleotides to mutate H3 and L3.

Affinity maturation *in vitro* is typically attempted by chain shuffling, or by random mutagenesis of the antibody CDRs and screening or selection for higher affinity variants. Either complete randomization or error-prone PCR is usually used as the mutational operator, but these are extremely inefficient probes for searching protein sequence space. To search protein sequence space as efficiently as possible we have developed a proprietary computer-assisted method for oligonucleotide-directed scanning mutagenesis, called Parsimonious Mutagenesis (PM; Balint and Larrick, 1993; Schier et al., 1996). The efficiency of PM is based on a rational reduction in the size of the sequence space which must be searched to one that can be completely encompassed in manageable libraries, and thoroughly searched with available screening techniques. This rational reduction of the sequence space is based on several observations. From the available X-ray

crystallographic data, between 15 and 22 residues of a protein antigen are typically in contact with a similar number of residues in the Ab combining site (Novotny et al., 1989; Laver et al., 1990; Novotny, 1991). However, energy calculations and mutational studies indicate that a subset of only ~30% of the contact residues, or 5-8 on average, contribute most of the binding energy (Novotny et al., 1989; Novotny, 1991; Mylvaganam et al., 1991; Tulip et al., 1992; Nuss et al., 1993). The remaining contact surface, therefore, presents multiple opportunities to develop additional high-affinity contacts, needing only a means to identify them. Consistent with the observation that most of the binding energy is concentrated in a few contacts, other studies have shown that affinities can often be profoundly altered by subtle changes in structure. For example, incremental contributions to binding energy of typical amino acid side chain groups have been measured as the comparative affinities of ligands which differ only by the presence or absence of the group. Such studies show that loss of a methyl group in a high-energy contact by replacement of Ala with Gly, Thr with Ser, or Ile with Val, could cost up to nearly three logs in binding energy. The same is true for the loss of a key carboxylate, as by replacement of Asp with Ala. Loss of a key hydroxyl by replacement of a Ser with Ala, or Tyr with Phe, could cost nearly six logs. In a hydrogen bond, gain or loss of 1 Å between proton donor and acceptor could be worth ~1 kcal or a factor of ~5 in affinity. These observations suggest that significant gains in antibody affinity may be achieved by a few key substitutions. To define a fully-accessible sequence space which is maximally enriched for sequences which contain new high-affinity contacts and retain all of the parental high-affinity contacts, we make use of two additional observations: (i) most high-affinity contacts are concentrated in the CDR3s, comprising 20-30 sites and (ii) the genetic code has evolved to maximize the frequency of adaptive substitutions among the 5-7 substitutions for each amino acid which may result from single base changes. Based on the foregoing we define the relevant sequence space for at least the first round of antibody affinity optimization as including all permutations of 1-5 mutations in up to 30 sites of the heavy and light chain CDR3s, and including all possible combinations of one-base amino acid

substitutions at each site. This comprises a sequence space of  $\sim 10^9$  different sequences. Using PM, it is possible to construct a library which would contain the entire sequence space in  $\sim 10^9$  clones, and using phage display it is possible to scan the entire library against immobilized antigen.

5 To assist with the construction of PM libraries, we have developed a computer program, called PMCAD, for the design of mutagenic oligonucleotides. The program computes optimum nucleotide (nt) mixtures for each position to be mutagenized in the oligonucleotides, based on the parent amino acid (aa) sequence, the user-selected mutation frequency distribution, and the desired sets  
10 of alternative amino acids. In this case we wish to maximize the abundance of 5-hit mutants so that all permutations with all possible combinations of up to 6 substitute aa at each site would be represented in a library of  $\sim 10^9$ . Such a library automatically contains all possible 6-aa combinations for all mutants with 1-4 hits also. A mutation frequency of 16.7% at each of 30 aa positions produces a  
15 binomial distribution in which 5-hit mutants comprise 19% of the library and mutants with 1-5 hits comprise 61% of the library. Substitute amino acid sets are controlled by using degenerate (doping) codons which may be selected to encode mixtures of more and less conservative analogs of the parent amino acid. NNT and NNG codons are particularly useful in this case because for most aa, they  
20 encode six different equally frequent 1-base codon changes to more and less conservative analogs. As is typical of doping codons in PM, the frequencies of 2- and 3-base codon changes is so low that the 1-base frequencies are only  $\sim 5\%$  below what they would be if they were the only substitutions.

We have previously shown that PM is particularly adept at identifying positions  
25 which can be improved most easily (Schier et al., 1996). Positions which were repeatedly altered in selected higher-affinity variants could be further improved by saturation mutagenesis. At such sites the parent residue may make a negative contribution to affinity which could be relieved by multiple alternatives. However, more intensive mutagenesis may be required to reveal the best alternative at each  
30 site. This is consistent with the contention of Novotny (1989, Biochem. 28, 4735; 1991, Molec. Immunol. 28, 201) that from crystal structures even the highest affinity

antibodies can be seen to make repulsive contacts with the antigen.

High affinity Mab fragments obtained by PM and expressed on the phage cell surface only constitute a very small fraction of the total cell population. We therefore use various enrichment procedures. These enrichment procedures are all based on functional recognition of the specific antigen by the Mab fragment expressed on the surface of the phage.

Selected putative high affinity Mab fragments are analyzed by BIAcore using B7.1ED and B7.2ED fusion proteins. Alternatively, additional PM primers are designed for the generation of new libraries, followed by a new round of selection for high affinity Mabs.

#### 6. Generation of BiTAbs, Diabody and Triabody molecules

The overall aim of the present invention is to generate a pharmaceutical composition that can simultaneously block the B7.1-CD28 and the B7.2-CD28 costimulatory pathways.

#### 7.1. Generation of BiTAbs (anti-B7.1/anti-B7.2, anti-B7.12/anti-B7.12) molecules

As an alternative to a diabody and a triabody molecule, a BiTAbs molecule can be constructed. A BiTAbs molecule has a less rigid structure, compared to a diabody and a triabody, and cross-links, and/or cross-reacts with the costimulatory molecules B7.1 and B7.2 expressed on the membrane of professional antigen-presenting cells, leading to the inhibition of antigen-specific T cell activation.

A BiTAbs molecule binds in a bivalent fashion to B7.1 or B7.12 on the one hand and to B7.2 or to B7.12 on the other hand. The binding affinity of the individual scFv's will be maturated using Parsimonious Mutagenesis (see above). Together with the divalent binding properties of this molecule we end up with a molecule with a very slow off-rate, being an ideal blocking agent for the application *in vivo*.

The intrinsic affinity of the different binding domains of the antibody constructs for their respective antigens is of minor importance, as both antigens normally appear

as membrane bound components thus promoting multivalent binding of the construct if the antigens are present on the same cell surface.

A BiTAB is a Bispecific Tetravalent Antibody molecule. The molecule consists of 4 scFv's; two anti B7.1 scFv's and two anti B7.2 scFv's (tetravalency). One single

5 BiTAB is a homodimer of two identical molecules, each containing both an anti B7.1 and anti B7.2 scFv (bispecificity). An anti B7.1 and an anti B7.2 scFv are linked together using a dimerisation domain, which drives the homodimerisation of the molecule (see figure 14). The VH and VL regions of the anti B7.1 and anti B7.2

10 Mab's are cloned from their respective hybridoma's by RT-PCR with a set of degenerate primers, for example the ones used in the Pharmacia RPAS Mouse scFv module. In a second PCR, both VH and VL are linked using a short synthetic linker. After cleavage with the appropriate restriction sites, the scFv coding sequences are ligated in a scFv expressionvector such as pCANTAB5E

15 (Pharmacia). To select for functional scFv's, phages will be generated after ligation in pCANTAB5E. These phages are panned against B7.1 or B7.2 positive cell lines. Binding phages are screened for their binding capacity in a FACS analysis with the respective B7.1 or B7.2 positive cell-lines. Correct binders are selected for further DNA sequence analysis. Anti B7.1 (B7-24) and B7.2 (1G10)

scFv's are used as building blocks to generate the B7.1/B7.2 BiTABs using 20 standard recombinant DNA techniques. A single BiTAB subunit starts with an anti-B7.1 scFv or an anti-B7.2 scFv followed by a dimerisation domain flanked by flexible linkers. The dimerisation domain in its turn links C-terminally to the anti-B7.2 scFv or the anti-B7.1scFv. Finally a detection and purification tag is added at the extreme C-terminus of the molecule. The sequence coding for the dimerisation

25 domain and the flanking linkers is made synthetically using the method described by Stemmer *et al.* (1995). Herefor we considered the optimal codon usage for

*E.Coli*-expression. This synthetic domain is subsequently linked to both the anti B7.1 and anti B7.2 (with tag) scFv's. As linkers between the dimerisation domain and the scFv's we use either the widely used (G<sub>4</sub>S)<sub>3</sub> sequence (Hoogenboom *et al.*,

30 1991) or the flexible and proteolysis-resistant truncated human IgG3 upper hinge region (Pack & Plückthun, 1992). As dimerisation domain we used a leucine zipper

type of domain (Kostelny et al., 1992; de Kruif & Logtenberg, 1996) or the helix-turn-helix motif described by Pack et al. (1993). An alternative dimerisation domain whereby a possible immunogenic reaction is reduced/avoided, is the JEM-1-peptide. This human peptide shows a 'leucine zipper' dimerisation motif with limited homology to Fos/Jun proteins (Duprez et al. 1997). A Cysteine could be included to enhance stability. As the C-terminal detection and purification tag we use a hexahistidine sequence. For immunogenic reasons we decided to make BiTab molecules both with or without a His6 tag. The sequences are assembled in such a way that functional domains are easily replaceable using unique restriction sites present in the molecule.

The BiTab coding sequence is inserted in an *E.coli* expressionvector with an appropriate secretion signal such as pelB or ompA signal sequence and is placed under control of an inducible promotor such as  $P_{lac}$ ,  $P_{trc}$  or  $P_L$ .

The BiTab expressionplasmid is introduced into suitable *E.coli* expressionstrains such as JM83 or HB2151. Expression levels in the *E.coli* periplasm and extent of dimerisation is analysed using small-scale expression experiments. Correctly dimerised BiTab molecules are purified from the *E.coli* periplasm using IMAC chromatography.

The DNA sequence and the protein sequence of the described BiTabB7-24-1G10H6 are represented in figure 15 and 16, respectively and the sequences of BiTab1G10-B7-24H6 in figure 17 and 18, respectively.

#### 7.1.1. Practical information

7.1.1.1. Single-step assembly of the dimerisation domain consisting of a helix-turn-helix domain flanked by the truncated human IgG3 hinge region as a flexible linker: HDH-domain (figure 19 and 20).

Equal amounts (10pmoles) from each of the 10 oligo's (primer 7624 to 7633) were combined and the mixture was subsequently diluted 100-fold in 50µl PCR mix containing 10mM Tris-HCl pH9/ 2.2mM MgCl<sub>2</sub>/ 50mM KCl/ 0.2mM each dNTP / 0.1%

TritonX-100/ 1U of Taq polymerase/ 0.1U of Pfu polymerase. The PCR program consisted of 35 cycles at 50°C for 30s. The obtained gene was amplified in a second PCR reaction. The gene assembly reaction mixture was diluted 40-fold in 100  $\mu$ l PCR mix containing 10mM Tris-HCl pH9/ 2.2mM MgCl<sub>2</sub>/ 50mM KCl/ 0.2mM each dNTP / 0.1% TritonX-100/ 5U of Taq polymerase/ 0.1U of Pfu polymerase/ 2 outside primers (primers 7622 and 7623) at a concentration of 1  $\mu$ M. The PCR program consisted of 23 cycles at 48°C for 30s. The gene-amplified DNA was purified and ligated in the pGEM-T-vector. The assembled DNA sequence was subjected to DNA sequence analysis.

#### List of used primers:

7622: 5' -CGCGCTCGAGATCAAACGGACC- 3'

7623: 5' -CGCGGAATTCGCGTTCGCGACTAG - 3'

7624: 5' -CGCGCTCGAGATCAAACGGACCCCGCTGGGTGATACCACTC- 3'

7625: 5' -CAGTTCACCTCCGGAGGTATGAGTGGTATCACCCAGCGGG- 3'

7626: 5' -ATACCTCCGGAGGTGAACTGGAAGAGCTGTTGAAACATCT- 3'

7627: 5' -GACCTTTCAGCAGTTCCTTCAGATGTTTCAACAGCTCTTC - 3'

7628: 5' -GAAAGAACTGCTGAAAGGTCCGCGGAAAGGTGAACTGGAG - 3'

7629: 5' -TTCAGGTGCTTCAGCAATTCCTCCAGTTCACCTTTCGCG - 3'

7630: 5' -GAATTGCTGAAGCACCTGAAAGAGCTGTTGAAAGGTACCC - 3'

7631: 5' -ATGGGTAGTATCACCCAGGGGGGTACCTTTC AACAGCTCT - 3'

7632: 5' -CCCTAGGTGATACTACCCATAACCAGCGGTCAGGTGCAACT - 3'

7633: 5' -CGCGGAATTCGCGTTCGCGACTAGTTGCACCTGACCGCTGGT-

3'

7.1.1.2. Introduction of an appropriate restrictionsite just before the start of the VH-domain of the gene encoding the scFvB7-24 or scFv 1G10.

To obtain a perfect in-phase fusion of the gene encoding the C-terminal scFvB7-24 or scFv1G10 to the gene encoding the HDH-domain in the gene encoding BiTabB7-



24-1G10H6 or BiTab1G10-B7-24H6 respectively, a *SpeI* restriction site was introduced just before the start of the gene encoding the VH-domain of the gene encoding scFvB7-24 and scFv1G10. This is done by PCR with the appropriate primers (primers 8067 and 8075). The PCR fragment was ligated in the pGEM-T vector and subjected to DNA sequence analysis.

List of used primers:

8067: 5' -GGACTAGTTCAGGTGCAGCTACAGCAGTCTG -3'

8075: 5' -GCCAGTGAATTCTATTAGTGGTGATG -3'

#### 7.1.1.3. Construction of BiTabB7-24-1G10H6 subunit

For the construction of BiTABB7-24-1G10H6 subunit, three fragments were ligated in-phase, namely the gene encoding the N-terminal scFvB7-24, the gene encoding the HDH-domain and the gene encoding the C-terminal scFv1G10H6. In that way the gene encoding BiTabB7-24-1G10H6 subunit, preceded by the pelB secretion signal, was cloned in an *E.coli* expression vector under control of the lac-promotor.

Fragment 1: pscFvB7-24H6 cleaved with *XhoI*-*EcoRI*

Fragment 2: pGEM-THDH cleaved with *XhoI*-*SpeI*

Fragment 3: pGEM-TscFv1G10s-e cleaved with *SpeI*-*EcoRI*

This expression plasmid pBiTabB7-24-1G10H6 was transformed in the expression strain JM83.

#### 7.1.1.4. Expression of BiTabB7-24-1G10H6 in JM83.

An overnight culture of pBiTabB7-24-1G10H6 in JM83 was 20x diluted in LB+100µg/ml amp+ 1% glucose and incubated at 28°C until an OD600 of 0.5-0.6 was reached. After removing the glucose of the medium, the culture was induced with 0.1mM IPTG and further incubated at 28°C during ±18h. Periplasmic fractions were prepared as explained before. Only a very small amount was secreted into the periplasm and almost all the expressed protein formed insoluble cytoplasmic

inclusion bodies. This was confirmed by NH<sub>2</sub>-terminal sequence analysis whereby the pelB secretion signal was still observed. This means that BiTabB7-24-1G10H6 couldn't be well secreted into the periplasm. Therefor, we cloned the gene encoding BiTabB7-24-1G10H6 and the gene in the opposite sense, encoding  
5 BiTab1G10-B7-24H6 in the pIGRI2-expressionvector (Innogenetics) without the pelB-secretion signal. This means that we are purifying BiTab-molecules from cytoplasmic inclusion bodies .

7.1.1.5. Construction of the gene encoding BiTabB7-24-1G10H6 without a secretion  
10 signal.

By PCR with specific primers 8299 and 8301, the gene encoding BiTabB7-24-1G10H6 was isolated in such a way that the pelB secretion signal was removed and that specific sites were created for ligation in the *E.coli* expressionvector  
15 pIGRI-2. The obtained PCR fragment was ligated in the pBSK(+) and subjected to DNA sequence analysis. For the construction of pIGRI-2BiTabB7-24-1G10H6, three fragments were isolated and fused in-phase.

1. pIGRI-2: blunted *NcoI* + *Sall*

.2. pBSKBiTabB7-24-1G10H6: blunted *SapI* + *BspEI*

20 3. PBSKBiTABB7-24-1G10H6: *BspEI* + *Sall*

By ligation of the three fragments, the gene encoding BiTabB7-24-1G10H6 without a secretion signal was cloned under control of the pL-promotor and the obtained plasmid was transformed to the *E.coli* expressionstrain MC1061(pAcI), SG4044(pAcI), UT5600(pAcI). After induction, cytoplasmic inclusion bodies will be  
25 formed and purification from inclusion bodies will be performed.

List of used primers:

8299: 5' -GGCCGCTCTTCGCAGCTACAGGAGTCTGG -3'

8301: 5' -CGACGTCGACTATTAGTGGTGATGGTG -3'

7.1.1.6. Single-step assembly of JEM-1 peptide using the method of Stemmer et al. (Figure 21 and 22)

This human peptide shows a 'leucine zipper' dimerisation motif with limited  
5      homology to Fos/Jun proteins.

Equal amounts (10pmoles) from each of the oligo's (primers 8528 to 8534) were  
combined and the mixture was subsequently diluted 100-fold in 50µl PCR mix  
containing 10mM Tris-HCl pH9/ 2.2mM MgCl<sub>2</sub>/ 50mM KCl/ 0.2mM each dNTP / 0.1%  
TritonX-100/ 1U of Taq polymerase/ 0.1U of Pfu polymerase. The PCR program  
10      consisted of 30 cycles at 52°C for 30s. The obtained gene was amplified in a second  
PCR reaction. The gene assembly reaction mixture was diluted 40-fold in 100 µl  
PCR mix containing 10mM Tris-HCl pH9/ 2.2mM MgCl<sub>2</sub>/ 50mM KCl/ 0.2mM each  
dNTP / 0.1% TritonX-100/ 5U of Taq polymerase/ 0.1U of Pfu polymerase/ 2 outside  
primers (primers 8526 and 8527) at a concentration of 1 µM. The PCR program  
15      consisted of 30 cycles at 50°C for 30s. The gene-amplified DNA was purified and  
ligated in the pGEM-T-vector. The assembled DNA sequence was subjected to  
DNA sequence analysis.

The dimerisation domain HDH in the BiTab molecule will be replaced by this  
human dimerisation domain to reduce immunogenicity.

20      List of used primers:

8526: 5' -CGCGTCCGGAGACCTGCAGTAC -3'

8527: 5' -CGCGCCTAGGGGGGTCTGTTC -3'

8528: 5' -CGCGTCCGGAGACCTGCAGTACCACTCGAACGTC -3'

25      8229: 5' -CGCGCCTAGGGGGGTCTGTTCAGACAGCTGCG -3'

8230: 5' -TGGCGCGTGAAAAAACCAGCTGATCCTGGAAAACGAAGC -3'

8231: 5' -GCTGGGTCGTAACACCGCGCAGCTGTCTGAACAGACCCCC -3'

8232: 5' -CGGTGTTACGACCCAGCGCTTCGTTTTCCAGGATCAGCTG -3'

8233: 5' -GTTTTTTTCACGCGCCAGACGTTTGAAGTGGTACTGCAGG -3'

30      8234: 5' -CGCCGCCCTAGGGGGGTCTGTTCAGACAGCTGCG -3'

The functional affinity of the BiTAbs constructs is measured using surface plasmon resonance (SPR) analysis measured with an optical biosensor (BIAcore from Pharmacia). As it will be difficult to mimic the density, the proportion, the distribution and the membrane diffusion capacity of the 2 antigens during the Biacore measurements, a qualitative analysis of the binding of these constructs is performed on the 3 antigens separately and on combinations of fixed concentrations of the 2 molecules. The binding curves are compared with the binding curves obtained with the monospecific antibody constructs (anti-B7.2 antibody, anti-B7.1 antibody or anti-B7.12 scFv). These results indicate whether the BiTAbs constructs have an advantage over monospecific holoantibodies with respect to their potential membrane binding. The final evaluation is done on the basis of the immunophysiological properties of these molecules.

## 7.2. Generation of diabody molecules (anti-B7.1/anti-B7.2 and anti-B7.12/anti-B7.12)

Diabodies are dimeric antibody fragments. In each polypeptide, a heavy-chain variable domain ( $V_H$ ) is linked to a light-chain variable domain ( $V_L$ ) but unlike scFv's, each antigen-binding site is formed by pairing of one  $V_H$  and one  $V_L$  domain from the two different polypeptides. This is achieved by shortening the linker between the  $V_H$  and  $V_L$  domains in each molecule (Holliger et al., 1993). Since diabodies have two antigen-binding sites they can be bispecific.

Mono- or bi-specific bivalent molecules are generated by shortening the flexible linker sequence between the  $V_H$  and  $V_L$  of the anti-B7.1 scFv B7-24, the anti-B7.2 scFv 1G10 and the scFv molecule with dual specificity for B7.1 and B7.2 (B7.12) to between five and ten residues (Gly4Ser to (Gly4Ser)<sub>2</sub>) and for the bi-specific molecules by cross-pairing the variable heavy and light chain domains from the two scFv's with different antigen recognition (B7.1/B7.2 and B7.12/B7.12). As a first trial we started the construction of monospecific diabodies B7-24 and 1G10 (figure 23,24 and 25,26). As an example for the different steps involved in such a construction, we have documented the construction of a anti-B7.1 diabody B7-24.

By PCR with specific primers the linker in scFvB7-24 was reduced to 5 or 10 aminoacids. Herefor, the VH domain of scFvB7-24 was amplified with primers 8218 and 8070( 5aa) ,and the VL domain of scFvB7-24 with primers 8073 and 8075 . In this process a *SapI* restriction site was introduced at the N-terminus of the VH-domain and a *EcoRI* restriction site was introduced at the C-terminus of the VL domain.  
Both PCR fragments were annealed and amplified by making a mix of equal amounts of both fragments, primers 8218 and 8075, 200 $\mu$ M dNTP, 1U Pfu-polymerase and 10x Pfu-buffer. The annealed fragment of 804 bp was ligated in the pBSK(+) and subjected to DNA sequence analysis.

The gene scFvB7-24L5H6, preceded by the pelB-secretion signal, was cloned in the pTrc99A expressionvector under control of the IPTG inducible pTrc-promotor and transformed to the JM83 *E.coli* expressionstrain. This was done by cleavage of pBSKB7-24L5H6 with *SapI*, followed by T4polymerase and *EcoRI* . The vector was cleaved with *NcoI* , followed by T4polymerase and *EcoRI*. Both fragments were ligated to obtain the expressionvector pTrcB7-24L5H6.

The same procedure was used for the construction of the three other expressionplasmids using the appropriate primers, namely pTrcB7-24L10H6 (primers 8218, 8071, 8074 and 8075), pTrc1G10L5H6 (primers 8218, 8070, 8073 and 8075) and pTrc1G10L10H6(primers 8218, 8071, 8074 and 8075). A Cystein could be included to enhance stability.

All 4 expressionplasmids were transformed to JM83. An overnight culture was 20x diluted in LB+100 $\mu$ g/ml *amp*+ 1% glucose and incubated at 28°C until an OD600 of 0.5 was reached. After removing the glucose of the medium, the culture was induced with 0.1mM IPTG and further incubated at 28°C during  $\pm$  18h. Periplasmic fractions were prepared as explained before. Only a small amount (10-30%) was secreted into the periplasm

List of used primers:

8218:  
5'-GGCCGCTCTTCGAAATACCTATTGCCTACGGCAG-3'

8070:

5'CTGAGTGAGCTCGATGTCCGATCCGCCACCGCCTGAGGAGACGGTGACCGT  
GGTC -3'

8071:

5 5'CTGAGTGAGCTCGATGTCCGATCCGCCACCGCCAGAGCCACCTCCGCCTGA  
GGAGACGGTGACCGTGGTC -3'

8073:

5'GTCACCGTCTCCTCAGGCGGAGGTGGCTCTGACATCGAGCTCACTCAGTCTC  
C -3'

10

8074:

5'GTCACCGTCTCCTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACAT  
CGAGCTCACTCAGTCTCC -3'

8075:

5' -GCCAGTGAATTCTATTAGTGGTGATG -3'.

15

The produced bivalent monospecific scFv B7.24 diabody molecules are purified from the periplasmic extract .

The bispecific diabodies (figure 27,28,29,30) are constructed oin a simililar way as described for the monospecific diabodies.

20

7.3. Generation of triabody molecules (B7.1/B7.1/B7.2 or B7.1/B7.2/B7.2 or  
B7.12/B7.12/B7.12)

25

The diabody construct can target only two molecules being B7.1 and B7.2 or B7.12 and B7.12 on the APC. A construct, which could target three molecules, e.g two B7.2 and one B7.1 molecule involved in this costimulatory pathway, namely a triabody, is aimed in this invention. A triabody is a mono-, a bi- or a trispecific molecule recognizing simultaneously e.g two B7.2 and one B7.1 molecules. Similar  
30 to the diabody, the triabody is a molecule with a rigid structure that prevents simultaneously binding to the three targets and so prevents the activation of the

APC. Each antigen-binding site is formed by pairing of one  $V_H$  and one  $V_L$  domain from the same or from two different polypeptides. This is achieved by shortening the linker between the  $V_H$  and  $V_L$  domains in each molecule to between -1 to 5 aa, preferable to 0 or -1 (referring numbering of Kabat). Additional association domains or disulfide bridges are incorporated in order to drive triabody formation as well as to maintain stability. As this construct can only bind monovalently to the target molecules, it means that, as for the diabody construct, the binding of the ScFv components in the triabody construct must be of high affinity. It is therefore important to increase the binding affinity of the individual ScFv's (see also diabody).

As a first trial we started the construction of monospecific triabodies B7-24 and 1G10 (figure 31,32 and 33,34). As an example for the different steps involved in such a construction, we have documented the construction of a anti-B7.1 triabody B7-24. By PCR with specific primers the linker in scFvB7-24 was removed. Herefor, the  $V_H$  domain of scFvB7-24 was amplified with primers 8218 and 8069, and the  $V_L$  domain of scFvB7-24 with primers 8072 and 8075. In this process a Sapl restriction site was introduced at the N-terminus of the  $V_H$ -domain and a EcoRI restriction site was introduced at the C-terminus of the  $V_L$  domain. Both PCR fragments were annealed and amplified by making a mix of equal amounts of both fragments, primers and , 200 $\mu$ M dNTP, 1U Pfu-polymerase and 10x Pfu-buffer. The annealed fragment of bp was ligated in the pBSK(+)-vector and subjected to DNA sequence analysis.

The gene scFvB7-24L0H6, preceded by the pelB-secretion signal, was cloned in the pTrc99A expressionvector under control of the IPTG inducible pTrc-promotor. This was done by cleavage of pBSKB7-24L0H6 with Sapl, followed by T4polymerase and EcoRI. The vector was cleaved with NcoI, followed by T4polymerase and EcoRI. Both fragments were ligated to obtain the expressionvector pTrcB7-24L0H6. The same procedure was used for the construction of the gene encoding the subunit of the monospecific triabody 1G10 using the appropriate primers 8218, 8069, 8072 and 8075.

Both expressionplasmids, pTrcB7-24L0H6 and pTrc1G10L0H6 were transformed to

JM83. An overnight culture was 20x diluted in LB+ 100µg/ml amp+ 1% glucose and incubated at 28°C until an OD600 of 0.5 was reached. After removing the glucose of the medium, the culture was induced with 0.1mM IPTG and further incubated at 28°C during ±18h. Periplasmic fractions were prepared as explained before. Only  
5 a small amount (10-30%) was secreted into the periplasm

List of used primers:

8218: 5'-GGCCGCTCTTCGAAATACCTATTGCCTACGGCAG-3'  
10 8069: 5'-CTGAGTGAGCTCGATGTCTGAGGAGACGGTGACCGTGGTC-3'  
8072: 5'-GTCACCGTCTCCTCAGACATCGAGCTCACTCAGTCTCC-3'  
8075: 5'-GCCAGTGAATTCTATTAGTGGTGATG-3'

Most of the expressed proteins formed insoluble, periplasmic inclusion bodies.  
15 The bispecific triabodies are constructed in a similar way as described for the monospecific triabodies (figure 35,36 and37,38).

The multimeric behaviour of the purified and unpurified molecules is analyzed. The ability of the molecules to bind the different antigens is tested in ELISA, FACS, T  
20 cell proliferation assay and BIACORE as described under section 7.5.

#### *7.4 Purification of diabodie and triabodi molecules*

Purification of diabodies and triabodies from the ScFv B7-24 molecule out of the  
25 soluble periplasmic fraction occurred under native as well as denaturing conditions. The soluble periplasmic fraction corresponding with 20 to 30% of the expressed product was purified on Ni<sup>2+</sup>-IDA under native conditions. The bound material was eluted by a 40 mM Imidazole wash step and a 200 mM Imidazole elution. The 200 mM Imidazole elution was divided (based upon SDS-PAGE CBB  
30 staining) into 3 pools (P1, P2 and P3) for the ScFv M24 L5 and into 2 pools for the ScFvM24 L0 (P1 and P2). P1 from both constructs ( ScFvM24 L5 and L0) contained



the peak fractions with the highest protein concentration but also with the highest amount impurities. P2 (ScFvM24 L0) and P3 (ScFv M24 L5) contained the tailing fractions of the elution peak, low in protein concentration but with a higher ScFv purity. P2 from ScFvM24 L5 contained the fractions in between P1 and P3 with an intermediate purity. Elution fractions were not as pure compared to the earlier native purification performed with ScFvM24. This can be caused due to the application of  $\text{Ni}^{2+}$  instead of  $\text{Zn}^{2+}$  or due to the fact that more E.coli host proteins are co-induced and co-expressed in the culture of the dia- and triabodies compared to the ScFv M24 culture. P2 and P3 from ScFvM24 L5 and P2 from ScFvM24 L0 were analysed in FACS and ELISA and MLR. P3 (ScFvM24 L5) and P2 (ScFvM24 L0) being the fractions with the highest purity, based on SDS-PAGE CBB staining, were concentrated and injected onto a Superdex 200 PC 3.2/30 gelfiltration column (Pharmacia) and compared with the gelfiltration profile of ScFvM24 L15. On the gelfiltration profile of the ScFv M24 L5 (diabody) two important peaks could be detected: the one with highest absorbance eluting at the same place were ScFvM24 L15 eluted, being the elution place for a 27 kD protein, indication for monomeric material, the other one eluting at the elution place for a 54 kD protein, indicating that diabodies are formed. On the gelfiltration profile of the ScFvM24L0 also two important peaks could be detected but with a much lower resolution. Both peaks had approximately the same absorbance, the first eluted at the place for a 54 kD protein (diabody) and the second one eluted a fraction earlier than the elution place for a 27 kD protein. (Figure 39: gelfiltration profile of a : ScFvM24 L0, b : ScFvM24 L5 and c : ScFvM24 L15). No clear peak at the supposed elution place for a triabody +/- 80 kD could be detected at this applied concentration. In a second SEC run a 10fold more concentrated sample of ScFvM24 L5 and ScFvM24 L0 has been applied on the same column under identical conditions. A decrease in resolution was observed but also a peak corresponding with the expected retention volume time for a triabody (MW 80 kD) appeared. For all three chromatograms (ScFvM24 L15, L5 and L0) gelfiltration fractions 5 up to 17 were analysed in ELISA. Most of the expressed proteins formed insoluble, periplasmic inclusion bodies.

The multimeric behaviour of the purified molecules is analyzed. The ability of the purified molecules to bind the different antigens is tested in BIAcore and ELISA experiments as described under section 6.4.

## 5 7.5. Characterization of the diabody and triabody molecules

The ability of the purified and unpurified diabody and triabody molecules to bind their specific antigen is tested in ELISA using the B7.1ED and B7.2ED fusion proteins. The cell-binding properties of these molecules are determined in FACS  
10 experiments. The molecules are further analyzed for their capacity to block T cell-APC interactions.

Binding specificities of the unpurified monospecific scFvB7-24, B7-24 diabody (scFvB7-24 L5) and B7-24 triabody (scFvB7-24 L0) molecules and of the unpurified monospecific scFv1G10, 1G10 diabody (scFv1G10 L5) and triabody (scFv1G10 L0)  
15 molecules were analysed in ELISA experiments using the B7.1ED and B7.2ED fusion proteins. As the periplasmic preparations of scFv1G10 and 1G10 triabody (scFv1G10 L0) contained very low amounts of scFv as demonstrated on Western blot, these two extracts were firstly concentrated 3.5 times before analysis in ELISA. For the ELISA, rabbit anti-human IgG Fc fragment specific antibodies were coated  
20 on MaxiSorb plates at a concentration of 1mg/ml in PBS buffer for 2h at 37°C or overnight at 4°C followed by blocking with PBS 0.1% casein for 1h at 37°C. Subsequently, 100 µl of B7.1ED fusion protein (500ng/ml) or B7.2ED fusion protein (500ng/ml) in PBS 0.1% casein was added and incubated for 1h at 37°C. After three washes with PBS 0.05% Tween20, the different antibodies were added in a fourfold  
25 serial dilution in PBS 0.1% casein starting from 1/1 dilution for the unpurified recombinant antibody preparations and from 1 mg/ml for purified scFvB7-24 and for the B7-24 (positive control) and 1G10 mAbs (positive control). Crude periplasmic preparation of nontransformed JM83 cells was included as a negative control. After three washes with PBS 0.05% Tween20, wells with the recombinant  
30 antibodies were incubated with mouse anti-his monoclonal antibody at 1 mg/ml for 1h at 37°C followed by three wash steps with PBS 0.05% Tween20. Subsequently,

the plates were incubated for 1h at 37°C with biotinylated goat anti-mouse (IgG + IgM) antibodies at 1.5 mg/ml followed by five washes with PBS 0.05% Tween20 and incubation with peroxidase-conjugated streptavidin at 0.1 mg/ml for 1h at 37°C. Color was generated by incubation with TMB for 30' at RT. The reaction was stopped by addition of 50 ml/well of 2N H<sub>2</sub>SO<sub>4</sub> and the absorbance at 450 and 595 nm was measured in an ELISA reader. For detection of B7-24 and 1G10 binding, incubation with mouse anti-his monoclonal antibody was omitted whereas all other incubation steps using antibodies and peroxidase-conjugated streptavidin were identical. OD values listed out in the figures are corrected for the blank OD value (OD value obtained if no recombinant antibody, B7-24 or 1G10 mAb was included in the ELISA). Results (figure 40) show that the unpurified scFvB7-24, B7-24 diabodies (scFvB7-24 L5) and B7-24 triabodies (scFvB7-24 L0) were able to bind the indirectly coated B7.1ED fusion protein. Binding was specific to the B7.1ED fusion protein since no significant binding to B7.2ED fusion protein was detected. Unpurified scFv1G10, 1G10 diabody (scFv1G10 L5) and 1G10 triabody (scFv1G10 L0) were able to bind to indirectly coated B7.2ED fusion protein (figure 41) but not to the B7.1ED fusion protein, demonstrating that these scFv1G10 recombinant antibodies bind specifically to the B7.2ED fusion protein.

The unpurified monospecific scFvB7-24, B7-24 diabody and B7-24 triabody molecules were further analysed for their binding capacity to the B7-1 molecules on the membrane of mouse 3T6-CD32-B7.1 cells, which are transfected with the cDNA encoding human B7.1. The 3T6-CD32 cells and 3T6-CD32 cells transfected with the cDNA of human B7.2 (3T6-CD32-B7.2) were included as a negative control. In addition, binding of these molecules on B7.1 expressing EBV transformed human B cells, RPMI 8866 was evaluated. To this end, 3T6-CD32, 3T6-CD32-B7.1, 3T6-CD32-B7.2 and RPMI 8866 cells (5x10<sup>5</sup> cells/staining) were preincubated for 20' at 4°C in 50 ml FACS buffer (PBS supplemented with 5% inactivated FCS and 0.02% sodium azide) supplemented with 10% normal rabbit serum. The different antibodies were added in a twofold serial dilution starting from 1/1 dilution for the periplasmic preparations of scFvB7-24, B7-24 diabody (scFvB7-24 L5) and B7-24 triabody (scFvB7-24 L0) and from 1 mg/ml for purified scFvB7-24 (positive control)

and incubated for 20' at 4°C. Periplasmic preparation of nontransformed JM83 cells at the same dilutions as described above was included as a negative control. After three washes in FACS medium, cells were incubated with mouse anti-his monoclonal antibody at 2 mg/staining for 20' at 4°C followed by 3 washes with FACS buffer. Subsequently, cells were incubated with 1.4 mg/staining of fluorescein (FITC)-conjugated goat anti-mouse IgG Fc fragment specific antibodies for 20' at 4°C, followed by three wash steps and analysis of the cells for fluorescent staining using a FACScan instrument. Cells only incubated with mouse anti-his antibody followed by incubation with FITC-conjugated goat anti-mouse IgG Fc fragment specific antibodies were included as a negative control for staining. FACS results using the periplasmic preparations at a twofold dilution and the purified scFvB24 at 1 mg/ml are listed out in figure 42a-b-c. The thin line represents the negative control staining whereas bold lines represent staining with the different crude periplasmic preparations and with purified scFvB7-24. These results show that unpurified scFvB7-24, B7-24 diabodies (scFvB7-24 L5) and B7-24 triabodies (scFvB7-24 L0) bound to the two B7.1 expressing cell lines: 3T6-CD32-B7.1 cells and RMPI8866 cells. Binding of these recombinant antibodies was found to be specific for B7.1 as they did not bind to 3T6-CD32 and to 3T6-CD32-B7.2 cells, which do not express B7.1 (data not shown).

Binding specificities of the semi-purified monospecific B7-24 diabody (scFvB7-24 L5 fraction p2 and p3) and triabody (scFvB7-24 L0 fraction p2) were checked with two different ELISA settings using the B7.1ED and B7.2ED fusion proteins. In a first ELISA, rabbit anti-human Fc antibody was coated at a concentration of 1 µg/ml in PBS buffer for 2h at 37°C followed by blocking with PBS 0.1% casein for 1h at 37°C. Subsequently 100 µl of B7.1ED fusion protein (500ng/ml) was added and incubated for 1h at 37°C. Purified scFvB7-24 and semi-purified B7-24 diabody (scFvB7-24 L5 fraction p3) or B7-24 triabody (scFvB7-24 L0 fraction p2) molecules were added in a twofold serial dilution starting from 500 ng/ml to 7.5 ng/ml and incubated for 1h at 37°C. After three washes with PBS 0.05% Tween20, wells were incubated for 1h at 37°C with mouse anti-his monoclonal antibody followed by three washes and incubation for 1h at 37°C with biotinylated goat anti-

mouse (IgG + IgM) antibodies at 1.5 mg/ml. After five washes with PBS 0.05% Tween20, incubation with peroxidase-conjugated streptavidin at 0.1 mg/ml for 1h at 37°C was performed. Color was generated by incubation with TMB for 30' at RT.

The reaction was stopped by addition of 50 ml/well of 2N H<sub>2</sub>SO<sub>4</sub> and the

absorbance at 450 and 595 nm was measured in an ELISA reader.

Binding specificities of the semi-purified monospecific B7-24 diabody (fraction p2 and p3) and triabody (fraction p2) molecules were also evaluated in a second ELISA. In this ELISA, wells were coated with mouse anti-his monoclonal antibodies (1 mg/ml) in PBS for 2h at 37°C or overnight at 4°C followed by blocking with PBS

0.1% casein. Purified scFvB7-24 and semi-purified monospecific B7-24 diabody (scFvB7-24 L5 fraction p2 en p3) and B7-24 triabody (scFvB7-24 L0 fraction p2) molecules were added in a twofold serial dilution starting from 1 µg/ml to 15 ng/ml and incubated for 1h at 37°C. Subsequently 100 µl of B7.1ED fusion protein (500 ng/ml) or B7.2ED fusion protein (500ng/ml) (negative control) were added (1h at 37°C), followed by incubation with peroxidase-conjugated sheep anti-human Ig (whole antibody) at 1/1000 for 1h at 37°C. As substrate TMB was used. Plates were measured at 450 and 595 nm.

Results of the first ELISA setting (figure 43) show that the semi-purified fractions of B7-24 diabody (scFvB7-24 L5 p3) and of scFvB7-24 triabody (scFvB7-24 L0 p2) displayed binding to indirectly coated B7.1ED fusion protein, comparable with the binding of purified scFvB7-24. Similar results were obtained in the second ELISA setting. Here also, B7-24 diabody and B7-24 triabody display binding to B7.1ED fusion protein. The observed binding was specific as no significant binding was observed to the B7.2ED fusion protein.

These semi-purified monospecific B7-24 diabody (scFvB7-24 L5 fraction p2 and p3) and triabody (scFvB7-24 L0 fraction p2) molecules are further analysed for their binding capacity to B7-1 molecules on the membrane of B7.1 expressing EBV transformed human B cells, RPMI8866. Cells (5x10<sup>5</sup> cells/staining) were preincubated for 20' at 4°C in 50 µl FACS buffer (PBS supplemented with 5% inactivated FCS and 0.02% sodium azide) supplemented with 10% normal rabbit serum. Subsequently, the cells were incubated with fourfold serial dilutions of

purified scFvB7-24, semi-purified B7-24 diabody (scFvB7-24 L5 fraction p2 and p3) or semi-purified B7.24 triabody (scFvB7-24 L0 fraction p2), starting from 1 mg/ml for 20' at 4°C. After three washes with FACS medium, the cells were incubated with mouse anti-his monoclonal antibody at 2 mg/staining for 20' at 4°C followed by 3 washes. Subsequently, cells were incubated with biotinylated goat anti-mouse (IgG+IgM) antibodies at 1.5 mg/staining and 1mg/staining of R-phycoerythrin (PE)-conjugated streptavidin for 20' at 4°C. Cells were analysed for fluorescent staining using a FACScan instrument. Between each incubation step, cells were washed three times with FACS buffer. Cells only incubated with mouse anti-his antibody followed by incubation with biotinylated goat anti-mouse antibody and R-PE conjugated streptavidin were included as a negative control for staining. FACS results using 1mg/ml of purified scFvB7-24, semi-purified B7-24 diabody (scFvB7-24 L5 fraction p2 and p3) and semi-purified B7-24 triabody (scFvB7-24 L0 fraction p2) are listed out in figure 44 a-b. The thin line represents the negative control staining whereas bold lines represent staining with the different recombinant antibodies. Results show that the semi-purified B7-24 diabodies and B7-24 triabodies bind to the human B7.1 expressing RPMI8866 cells, in a comparable way as scFvB7-24.

The semi-purified constructs are further analyzed for their capacity to block T cell-APC interactions. T cells were purified out of whole heparinized blood on Ficoll-Paque (density 1.077, Pharmacia Biotech) density gradients. The peripheral blood mononuclear cells (PBMC) present in the interface were washed three times in 40 ml of RPMIbic supplemented with 10% inactivated FCS. Subsequently, monocytes were removed by cold aggregation (Mentzer et al., 1986). To this end, PBMC were resuspended in 40 ml of RPMIbic supplemented with 10% inactivated FCS and slowly rotated for 30' at 4°C. Monocyte aggregates were allowed to sediment over a 15' period incubation on ice, and the non-aggregated cells containing enriched T cells and B cells were carefully aspirated and centrifuged for 10' at 1200 rpm. T cells were further enriched using Lympho-Kwik-T (One lambda Inc, Los Angeles, CA). This reagents contains a mixture of anti-monocyte and anti-B cell mAbs and complement. Lymphocytes were resuspended in 3 ml Lympho-Kwik-T and the mixture was incubated for 45' at 37°C. Subsequently, cells

were resuspended in 0.5 ml of PBS and centrifuged for 2' at 2000 rpm and washed twice.

The mouse fibroblast cell line, 3T6, transfected with the cDNA encoding human B7.1 and human CD32 (3T6-CD32-B7.1) was mitomycin C treated. Cell pellet of 1  
5 subconfluent falcon was dissolved in 800 ml RPMIbic and 200 ml Mitomycin C (250 mg/ml) and incubated for 40' at 37°C followed by two washes. Subsequently, cells are suspended in 30 ml RPMIbic and incubated for 15' at 37°C followed by one additional wash step. In the mixed lymphocyte culture (MLR), the mitomycin C treated 3T6-CD32-B7.1 cells ( $10^4$  cells/well) were incubated with OKT3 (1 mg/ml) for  
10 1 h at 37°C followed by a 1 h incubation at 37°C with threefold serial dilutions of B7-24 mAb, recombinant purified scFvB7-24 or semi-purified B7-24 diabody (scFvB7-24 L5 fractions p2 and p3) or B7-24 triabody (scFvB7-24 L0 fraction p2), starting from 1  $\mu$ g/ml. Subsequently, purified T cells ( $5 \times 10^4$  cell/well) were added and incubated for 5 days. After 5 days, cells were incubated with 1 mCi ( $^3$ H)-thymidine for 6 h and  
15 harvested using an automated cell harvester. ( $^3$ H)-thymidine incorporation was determined with a liquid scintillation counter.

Results showed that the recombinant scFvB7-24 antibodies, the B7-24 diabody (figure 45) and B7-24 triabody (figure 46) molecules displayed neutralizing activities, comparable to those of the parent B7-24 mAb, as measured in MLR.

20 Gelfiltration fractions 5 to 17 of the B7-24 diabody (scFvB7-24 L5 p3) and B7-24 triabody (scFvB7-24 L0 p2) chromatography and fractions 10 to 17 of the scFvB7-24 chromatography were evaluated for specific binding in ELISA. To this end, mouse anti-his antibody (1 mg/ml) was coated in PBS for 2h at 37°C followed by blocking with PBS 0.1% casein for 1h at 37°C. The different gelfiltration fractions of  
25 scFvB7-24, B7-24 diabodies (scFvB7-24 L5 p3) and B7-24 triabodies (scFvB7-24 L0 p2) were added in twofold serial dilutions and incubated for 1h at 37°C followed by incubation with 100 ml of B7.1ED fusion protein (500 ng/ml) for 1h at 37°C.

Subsequently the wells were incubated with peroxidase-conjugated sheep anti-human Ig (whole antibody) at dilution 1/1000 for 1h at 37°C. Color was generated  
30 by incubation for 30' at RT with TMB. Plates were read at 450 and 595 nm. OD values listed out in the figures are corrected for the blank OD value (OD value

obtained if no recombinant antibody was included in the ELISA setting). These results confirm that B7-24 triabody (ScFvB7-24 L0) (figure 47, lower panel) displays reactivity in the triabody region as well as in the diabody region and the monomeric region, while B7-24 diabody (scFvB7-24 L5) (figure 47, middle panel) has reactivity in the diabody region and monomeric region. In contrast, scFvB7-24 (figure 12, upper panel) has only reactivity in the monomeric region. This confirms that scFvB7-24 L0 indeed has the capacity of forming trimers (triabodies) and dimers (diabodies), while ScFvB7-24 L5 has only the capacity of forming dimers (diabodies). ScFvB7-24 L15 remains monomeric.

## 8. Evaluation of the B7 blocking agents

### 8.1 Chemically cross-linked anti-B7 antibodies suppress the proliferation of human T cells and induce IL-12 production in peripheral blood mononuclear cells (PBMC)

As a proof of concept we wanted to test the immunosuppressive effect of chemical cross-linked anti-B7.1 mAb and anti-B7.2 mAb (further referred as anti-B7.1/B7.2 mAb). We followed the protocol described in 'Current protocols of Immunology' for 'Chemically cross linking antibodies with SPDP' (Current Protocols of Immunology, Supplement 14, Unit 2.13). Briefly, anti-B7.1 mAb (B7-24) and anti-B7.2 mAb (1G10) are cross-linked using the heterobifunctional compound N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP). This reagent binds randomly to  $\epsilon$ -amino groups on lysine residues and forms reducible disulfide bonds between antibodies. The use of a bifunctional cross linker is very important, because the intention here is to create a bifunctional antibody (recognizing two different antigens) and therefore only a cross linking between anti-B7.1 mAb (B7-24) and anti-B7.2 mAb (1G10) will be performed. The formation of 1G10-1G10 or B7-24-B7-24 is not desirable. The resulting bispecific molecules consists of aggregates of antibodies of varying size linked together at random sites. The bifunctional antibody was obtained by performing a size exclusion chromatography (Superdex



G 200) (SEC), which separates the dimeric antibody (300 kDa) from the monomeric antibodies (150 kDa). The gelfiltration resulted in 4 main peaks (figure 48). Peak 2 and 3 appeared at the suspected place for dimeric (B7-24/1G10 crosslinked mAbs) and monomeric mAbs (B7-24 mAb). Peak 1 eluted at the place for oligomers. As we have already seen that the purified 1G10 is retained much longer onto gelfiltration column, we can suppose the peak 3 and 4 are dimeric and monomeric 1G10.

Gelfiltration fractions 19, 20 and 21 (peak 2), containing dimeric B7-24/1G10 crosslinked mAbs were tested and compared with a mixture of anti-B7.1 mAb (B7-24)- and anti-B7.2 mAb (1G10) for their binding capacity to the human B7.1 and B7.2

molecules. Mouse 3T6 cells transfected with the cDNA encoding human CD32 and the human B7.1 molecule (3T6-CD32-B7.1) or encoding the human B7.2 molecule (3T6-CD32-B7.2) (De Boer et al., 1992) were incubated with fractions 19 (3 µg/cell pellet) and 20 (2 µg/cell pellet) of the cross-linked anti-B7.1/B7.2 mAb or, as control, with a mixture of anti-B7.1 mAb (B7-24) (0.5 µg/cell pellet) or anti-B7.2 mAb

(1G10) (0.5 µg/cell pellet). Cells (0.5-1x 10<sup>5</sup> cells/sample) were incubated for 15' at 4°C with the different mAbs. After washing twice in RPMI1640 supplemented with 10% FCS, the cells were incubated for another 15' at 4°C with goat anti-mouse antibodies conjugated to fluorescein isothiocyanate (FITC). The cells were washed twice in RPMI1640 supplemented with 10% FCS and finally suspended in PBS

supplemented with 1% BSA and 0.1% NaN<sub>3</sub> and analyzed with a FACScan flow cytometer (Becton Dickinson). The specific binding of the monoclonal antibodies is expressed as the mean fluorescence intensity in arbitrary units. The results (figure 49 a-b) showed that the chemical cross-linked anti-B7.1/B7.2 mAb binds both the human B7.1 molecule (3T6-CD32-B7.1 cells) and the human B7.2 molecule (3T6-CD32-B7.2 cells), indicating that the chemical cross-linked mAb exists of a B7.1 and a B7.2 recognizing part.

Gelfiltration fractions 19, 20 and 21 (peak 2), containing dimeric B7-24/1G10 crosslinked mAbs were tested and compared with a mixture of anti-B7.1 mAb (B7-24)- and anti-B7.2 mAb (1G10) for their binding capacity to the human B7.1 and B7.2 molecules in a coating ELISA with hB7.1ED and hB7.2ED fusion proteins. 500 ng of hB7.1ED and hB7.2ED fusion proteins were coated in PBS for 2h at 37°C followed by

blocking in PBS 0.1% casein for 1h at 37°C. Coating without B7ED fusion proteins was included as a negative control. Subsequently, two different dilutions (1/1000 and 1/10000) of above mentioned fractions were incubated for 1 h at 37°C. In this way, the amount of material added to the ELISA correlates approx. with 250 ng/ml and 25 ng/ml for the dimer fraction 20. Detection was done by incubation with goat anti-mouse IgG (Fcγ) HRP labeled antibody for 1 h at 37°C followed by the addition of TMB as substrate. Plates are read at 450-595 nm on a microtiter plate reader. Results showed that the B7-24/1G10 crosslinked monoclonal antibodies are able to bind both hB7.1 and hB7.2. No signal was detected on the blanco coating conditions.

The simultaneous binding of B7.1 and B7.2 to the cross-linked anti-B7.1/B7.2 mAb was also evaluated using SPR-analysis with the BIAcore instrument (Pharmacia Biosensor AB, Uppsala, Sweden). In this experiment, hB7.2ED fusion protein was immobilized directly onto a CM5 sensor chip using an amine coupling according to the manufacturer's procedure. Briefly, the sensor chip surface was initially activated with N-hydroxysuccinimide and N-ethyl-N'-(3-ethylaminopropyl)carbodiimide. A continuous flow of 5 µg/ml soluble hB7.2ED fusion protein was injected over the activated surface at pH 4.8. Residual unreacted ester groups were blocked with ethanolamine. This coupling procedure resulted in an immobilization level of about 3450 RU (resonance units). Onto this coupled B7.2Fc, the cross-linked B7-24/1G10 construct (pool 20) was injected and a specific interaction of about 1500 RU could be monitored (Figure 50). Subsequent injection of hB7.1ED fusion protein resulted in an increase of about 460 RU. These results clearly indicate that B7-24/1G10 preparation contains cross-linked constructs that recognize both B7.1 and B7.2. As the binding of the second B7 molecule (B7.1ED fusion protein) is fairly high, we can conclude that most 1G10 containing molecules are B7-24/1G10 cross-links.

The *in vitro* proliferation of CD3 - activated human T lymphocytes in the presence of B7.1 and B7.2 molecules expressed on the membrane of different antigen presenting cells (APC) is largely blocked by the combination of anti-B7.1 mAb and anti-B7.2 mAb. In our experiments, human peripheral blood mononuclear cells

(PBMC) were isolated from buffy coat by density centrifugation and cultured with anti-CD3 mAb (OKT3, 0.3  $\mu$ g/ml) in the presence of a mixture of anti-B7.1 mAb (B7-24, 0.5  $\mu$ g/ml) and anti-B7.2 mAb (1G10, 0.5  $\mu$ g/ml) or in the presence of the chemical crosslinked anti-B7.1/B7.2 mAbs (1  $\mu$ g/ml). Our results show that chemical crosslinked anti-B7.1/B7.2 mAbs are able to stronger inhibit this proliferation of activated human T lymphocytes, compared to the combination of the two mAbs. Thus, the chemical crosslinked anti-B7.1/B7.2 mAbs have a stronger immunosuppression potency than the combination an anti-B7.1 mAb and an anti-B7.2 mAb.

Activation of human PBMC, isolated from buffy coat by density centrifugation, by anti-CD3 mAb (OKT3, 0.3  $\mu$ g/ml) induces the production of IL-12 by the monocytes present in these cultures. This IL-12 production by the human PBMC cultures, is suppressed by the combination of anti-B7.1 mAb (B7-24) and anti-B7.2 mAbs (1G10). Chemical crosslinked anti-B7.1/B7.2 mAbs are able to stronger inhibit this IL-12 production, compared to the combination of the two mAbs. As IL-12 activates T lymphocytes and induces IFN- $\gamma$  release by Th-1 lymphocytes, this stronger inhibition by the crosslinked anti-B7.1/B7.2 mAbs, compared to the inhibition by the combination of the two mAbs alone, means indirectly a stronger inhibition of the T lymphocyte activation. Thus, again these experiments demonstrate a stronger immunosuppression potency of the chemical crosslinked anti-B7.1/B7.2 mAbs compared to the combination of an anti-B7.1 mAb and an anti-B7.2 mAb.

In a second proliferation experiments, the crosslinked monoclonal antibodies were further evaluated to block T cell-APC interactions in a MLR wherein T cells are cultured with RPMI 8877 cells, a EBV transformed B cell line expressing human B7.1 and human B7.2 molecules. T cells were purified out of whole heparinized blood on Ficoll-Paque (density 1.077, Pharmacia Biotech) density gradients. The peripheral blood mononuclear cells (PBMC) present in the interface were washed three times in 40 ml of RPMIbic supplemented with 10% inactivated FCS. Subsequently, monocytes were removed by cold aggregation (Mentzer et al., 1986). To this end, PBMC were resuspended in 40 ml of RPMIbic supplemented with 10% inactivated FCS and slowly rotated for 30' at 4°C. Monocyte aggregates were allowed to

sediment over a 15' period incubation on ice, and the non-aggregated cells containing enriched T cells and B cells were carefully aspirated and centrifuged for 10' at 1200 rpm. T cells were further enriched using Lympho-Kwik-T (One lambda Inc, Los Angeles, CA). This reagents contains a mixture of anti-monocyte and anti-B cell mAbs and complement. Lymphocytes were resuspended in 3 ml Lympho-Kwik-T and the mixture was incubated for 45' at 37°C. Subsequently, cells were resuspended in 0.5 ml of PBS and centrifuged for 2' at 2000 rpm and washed twice.

The EBV transformed B cell line, RPMI8877, was mitomycin C treated. Cell pellet of 1 subconfluent falcon was dissolved in 800 ml RPMIbic and 200 ml Mitomycin C (250 mg/ml) and incubated for 40' at 37°C followed by two washes. Subsequently, cells are suspended in 30 ml RPMIbic and incubated for 15' at 37°C followed by one additional wash step. In the mixed lymphocyte culture (MLR), the mitomycin C treated RPMI8877 cells ( $10^4$  cells/well) were incubated with OKT3 (1 mg/ml) for 1 h at 37°C followed by a 1 h incubation at 37°C with serial dilutions of crosslinked B7.24/1G10 mAbs (fraction 19 or 20) or with the combination of B7.24 (1 µg/ml) and 1G10 (1 µg/ml). Subsequently, purified T cells ( $5 \times 10^4$  cell/well) were added and incubated for 5 days. After 5 days, cells were incubated with 1 mCi ( $^3$ H)-thymidine for 6 h and harvested using an automated cell harvester. ( $^3$ H)-thymidine incorporation was determined with a liquid scintillation counter. Results showed (figure) that the crosslinked b7.24/1G10 monoclonal antibodies displayed neutralizing activities, comparable to those of the parent B7-24 mAb, as measured in MLR. Our results show that chemical crosslinked anti-B7.1/B7.2 mAbs are able to stronger or comparable inhibit this proliferation of activated human T lymphocytes, compared to the combination of the two mAbs. Thus, the chemical crosslinked anti-B7.1/B7.2 mAbs have a stronger or comparable immunosuppression potency than the combination an anti-B7.1 mAb and an anti-B7.2 mAb.

## 8.2 Induction of donor specific tolerance in a rhesus monkey skin transplant model

The immunosuppressive effect of the combined treatment with Cyclosporin A (CsA) and a monoclonal antibody directed against the B7.1 molecule (B7-24 mAb) is examined in a rhesus monkey skin transplant model. Animals receive the experimental medication B7.24 mAb(0.5 mg/kg) and CsA (5 mg/kg) for 10 days starting one day before the day of transplantation. Transplant rejection is monitored by scoring the skin grafts. At several time points during the experiment, blood samples are taken to determine the blood level of CsA, serum levels of the mAb anti-CD80, the rhesus monkey anti-mouse anti-body (RAMA) response, anti-donor antigen antibody response. Combined treatment of the anti-CD80 mAb and CsA in this rhesus monkey skin transplantation model results in increased skin graft survival time (control 10 days, treatment 14 days) but fails to induce donor specific tolerance. Thus, short term immunosuppression, resulting in prolonged skin graft survival can be obtained using CsA and B7-24 mAb as prophylactic treatment in rhesus monkey skin transplant model. Data has been accumulating that blocking both CD80 and CD86 molecules using or CTLA-4-Ig or a combination of anti-CD80 mAb and anti-CD86 mAb can induce often indefinite prolongation of allograft survival in rat and mouse cardiac transplantation models (Bashuda et al., 1996; Lenschow et al., 1992, 1995; Lin et al, 1993; Pearson et al., 1994). Therefore, it can be suggested that the combination of anti-B7.1 mAb and CsA in this rhesus monkey skin transplantation model only results in a prolongation of the graft survival time, but fails to induce donor-specific non-responsiveness, since the B7.2-CD28 pathway is not blocked.

### 8.3 Induction of donor specific tolerance in a rhesus monkey kidney transplant model

A combination of anti-B7.1 mAb, anti-B7.2 mAb and CsA seems to be an optimal treatment to induce donor-specific tolerance during allo-transplantation. Rhesus monkeys receive a kidney transplantation from an allogeneic donor mismatched for at least one MHC class I and one class II antigen. Graft survival time is indicative for the immunosuppressive potency of the therapy combining anti-B7.1

mAb, anti-B7.2 mAb and CsA. The mAbs (0.5 mg/kg at day -1, 0.25 mg/kg on day 0 till 12) are given daily for 14 days starting at day -1. The CsA (10 mg/kg) is given daily for 35 days starting at day 1. At several time points during the experiment, blood samples are taken to determine the blood level of CsA, serum levels of the mAbs anti-B7.1 and anti-B7.2, the rhesus monkey anti-mouse anti-body (RAMA) response, anti-donor antibody response.

In a pharmacokinetic-toxicology (PK-Tox) study, with the same design as the transplantation study, no signs of acute cytotoxicity of the anti-B7.1 mAb and anti-B7.2 mAb in combination with CsA are observed. In the skin transplantation study (combination of anti-B7.1 mAb and CsA), the anti-B7.1 concentrations decreases to undetectable values after 9 days due to a RAMA response. Using the combination of anti-B7.1 mAb, anti-B7.2 mAb and CsA, total mouse mAb serum concentrations remained high and are even detectable on day 20. No RAMA response can be detected in the monkeys in this PK-Tox study. Thus, combined treatment with anti-B7.1 mAb, anti-B7.2 mAb and CsA is able to prevent an immunological response against mouse mAbs, indicating the high immunosuppressive potency of this combination.

Rhesus monkeys, not receiving any treatment after a kidney graft transplantation, reject the graft 6 days after transplantation. Combined treatment of the animals with anti-B7.1 mAb and anti-B7.2 mAb strongly prolongs the kidney graft survival time (22 days and 35 days). Using this combined treatment with anti-B7.1 mAb and anti-B7.2mAb, the mouse mAb serum concentrations remains high and both anti-B7.2 mAb and anti-B7.1 mAb are still detectable on day 15. As long as the mAbs are in circulation, no or only a weak RAMA response can be measured. As the concentration of circulating mAbs become low, the RAMA responses become high and the immunosuppressive potency of this combination is lost. Longer treatment with the combination of anti-CD80 mAb and anti-CD86 mAb can enhance the immunosuppressive capacity of this combination. Also, combined treatment of the animals, after kidney transplantation, with anti-B7.1 mAb and the anti-B7.2 mAb together with CsA can enhance the immunosuppressive capacity.

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CLAIMS

1. A molecule which cross-links, or cross-reacts with, B7.1 and B7.2, and which does not comprise a variable domain of a monkey antibody or the extracellular domain of CTLA4 or CD28.
2. A molecule according to claim 1, wherein said molecule comprises at least one first domain which binds B7.1 or B7.2 or cross-reacts with B7.1 and B7.2, at least one second domain which binds B7.1 or B7.2 or cross-reacts with B7.1 and B7.2, and optionally a third domain which couples the first and the second domain(s).
3. A molecule according to claim 2, wherein said first and second domain is a polypeptide or a low-molecular-weight nonpeptide molecule.
4. A molecule according to claim 2, wherein said first and second domain is an anti-B7.1 antibody or an anti-B7.2 antibody or an antibody which cross-reacts with both B7.1 and B7.2, or, a humanized antibody, a single-chain fragment or another fragment thereof which has largely retained the specificity of said antibodies.
5. A molecule according to claim 2 wherein said third domain is a (poly)peptide, any chemical coupling agent or any oligomerization domain.
6. A molecule according to claim 4, wherein said anti-B7.1 and anti-B7.2 antibody are monoclonal antibody B7-24 or 5B5 and monoclonal antibody 1G10, 3H10, 5F3, 7B8, 9D8, 11B9, 13B9, 13D3 or 14F1, respectively.
7. A molecule according to claims 1 to 6 selected from the group consisting of miniantibodies, diabodies, trivalent antibodies, tetravalent antibodies, small antigen-binding peptides and low-molecular-weight nonpeptide molecules.
8. A molecule according to any of claims 1 to 7, wherein said cross-linking of B7.1

and B7.2 results in the inhibition of immuno-activating soluble mediators and/or the activation of immuno-inhibiting soluble mediators.

5 9. A molecule according to claim 8, wherein said immuno-activating soluble mediators are selected from the group consisting of IL-1, IL-6, IL-12 and TNF- $\alpha$ , and wherein said immuno-inhibiting soluble mediators are selected from the group consisting of IL-10, TGF- $\beta$  and prostaglandins.

10 10 A method for producing a molecule according to any of claims 1 to 9.

11. A pharmaceutical composition comprising a molecule according to any of claims 1 to 9 in a pharmaceutically acceptable excipient.

15 12. A molecule according to any of claims 1 to 9 or a composition according to claim 11 for use as a medicament.

20 13. A molecule according to any of claims 1 to 9 or a composition according to claim 11, possibly in combination with immunosuppressive agents, for use in inhibiting antigen-specific cell activation.

25 14. A molecule according to any of claims 1 to 9 or a composition according to claim 11, possibly in combination with immunosuppressive agents, for use in preventing or treating diseases of the immune system, in particular for preventing or treating graft rejection, graft versus host disease, allergy and autoimmune diseases.

30 15. A molecule according to claims 13 or 14 wherein said immunosuppressive agent is chosen from the group consisting of cyclosporin A; FK 506; rapamycin; OKT-3; OKT-4; SB-210396; T10B9; BTI 322; Mycophenolate mofetil; anti-thymocyte globulin; anti-lymphocyte immunoglobulin; the combination of cyclosporin A, azathioprine and glucocorticosteroids; azathioprine; leflunomide; adenosin

deaminase inhibitor; purine nucleoside phosphorylase inhibitor; MHC-peptide and IL-2 receptor mAb.

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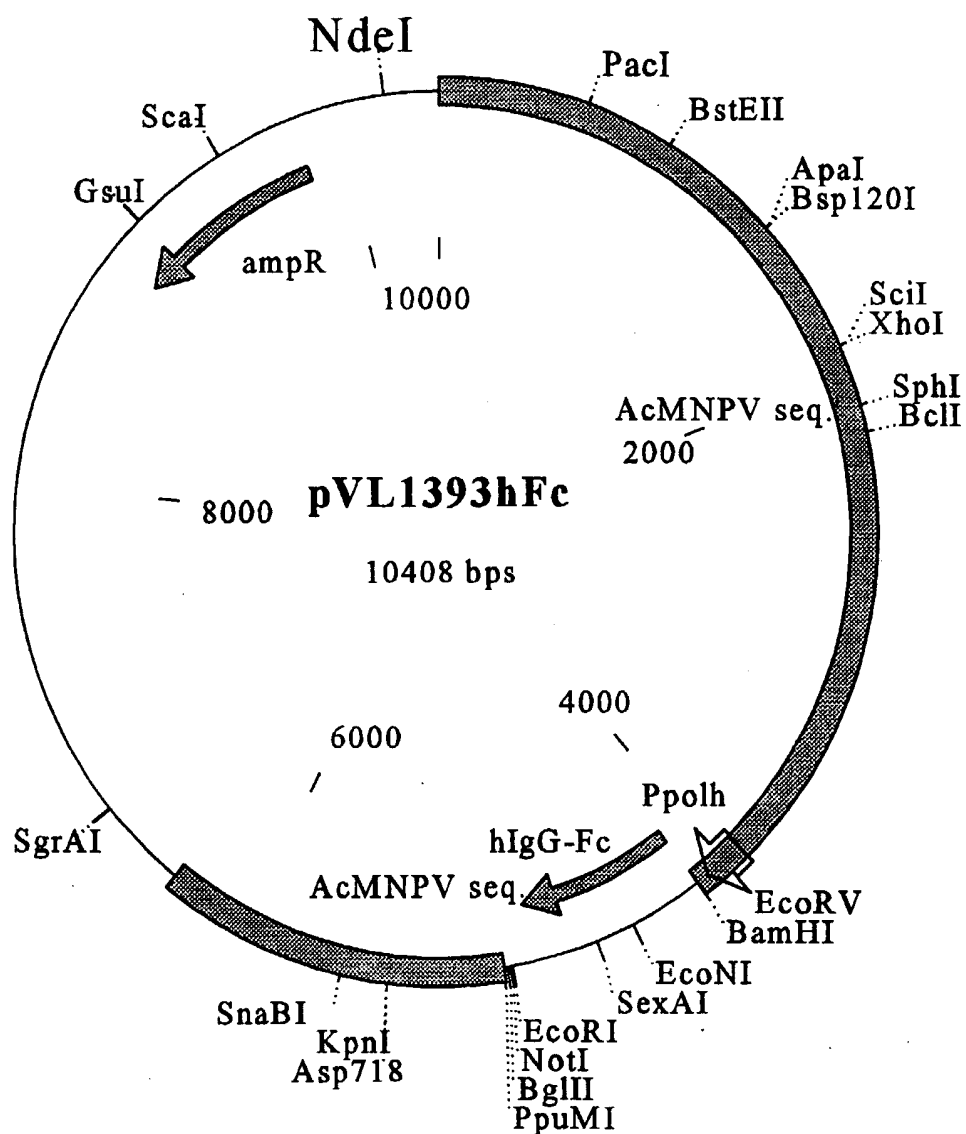


Figure 1



Figure 2

1 MGHTRRQGTSPSKCPYLNFFQLLVLAGLSHFCSGVIHVTKEVKEVATLSC  
51 GHNVSVEELAQTRIYWQKEKKMVLTMMSGDMNIWPEYKNRTIFDITNNLS  
101 IVILALRPSDEGTYECVVLKYEKDAFKREHLAEVTLSVKADFPTPSISDF  
151 EIPTSNIRRIICSTSGGFPEPHLSWLENGEELNAINTTVSQDPETELYAV  
201 SSKLDFNMTANHSFMCLIKYGHRLRVNQTFNWNTTKQEHFPDKRSPGLQEP  
251 KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVS  
301 HEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK  
351 EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTC  
401 LVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRW  
451 QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

(SEQ ID NO 49)

Number of residues : 480

Molecular weight (MW) : 54298

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Figure 3

1 MGLSNILFVMAFLLSGAAPLKIQAYFNETADLPCQFANSQNQSLSELVVF  
51 WQDQENLVLNEVYLGKEKFDVHSHKYMGRTSFDSDSWTLRLHNLQIKDKG  
101 LYQCIHHKKPTGMIRIHQMNSELSVLNFSQPEIVPISNITENVYINLT  
151 CSSIHGYPEPKKMSVLLRRTKNSTIEYDGIMQKSQDNVTELYDVSISLSVS  
201 FPDVTSNMTIFCILETDKTRLLSSPFSIELEDPQPPPDHRSPGLQEPKSC  
251 DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED  
301 PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK  
351 CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK  
401 GFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQG  
451 NVFSCSVMHEALHNHYTQKSLSLSPGK

(SEQ ID NO 50)

Number of residues : 477

Molecular weight (MW) : 53827

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Figure 4

1     MGHTRRQGTSPSKCPYLNFFQLLVLAGLSHFCSGVIHVTKEVKEVATLSC  
51     GHNVSVEELAQTRIYWQKEKKMVLTMMSGDMNIWPEYKNRTIFDITNNLS  
101    IVILALRPSDEGTYECVVLKYEKDAFKREHLAEVTL SVKADFPTPSISDF  
151    EIPTSNIRRIICSTSGGFPEPHLSWLENGEELNAINTTVSQDPETELYAV  
201    SSKLDFNM TTNH SFMCLIKYGH LRVNQTFNWNTTKQOHFPDNEEEEYMPME

(SEQ ID NO 51)

Number of residues : 251.

Molecular weight (MW) : 28768

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Figure 5

1 MGLSNILFVMAFLLSGAAPLKIQAYFNETADLPCQFANSQNQSLSELVVF  
51 WQDQENLVLNEVYLGKEKFDSVHSKYMGRTSFDSDSWTLRLHNLQIKDKG  
101 LYQCIHHKKPTGMIRIHQMNSELSVLNFSQPEIVPISNITENVYINLT  
151 CSSIHGYPEPKMSVLLRTKNSTIEYDGIMQKSQDNVTELYDVSISLSVS  
201 FPDVTSNMTIFCILETDKTRLLSSPFSIELEDPQPPPDHHHHHH  
(SEQ ID NO 52)

Number of residues : 244

Molecular weight (MW) : 27799

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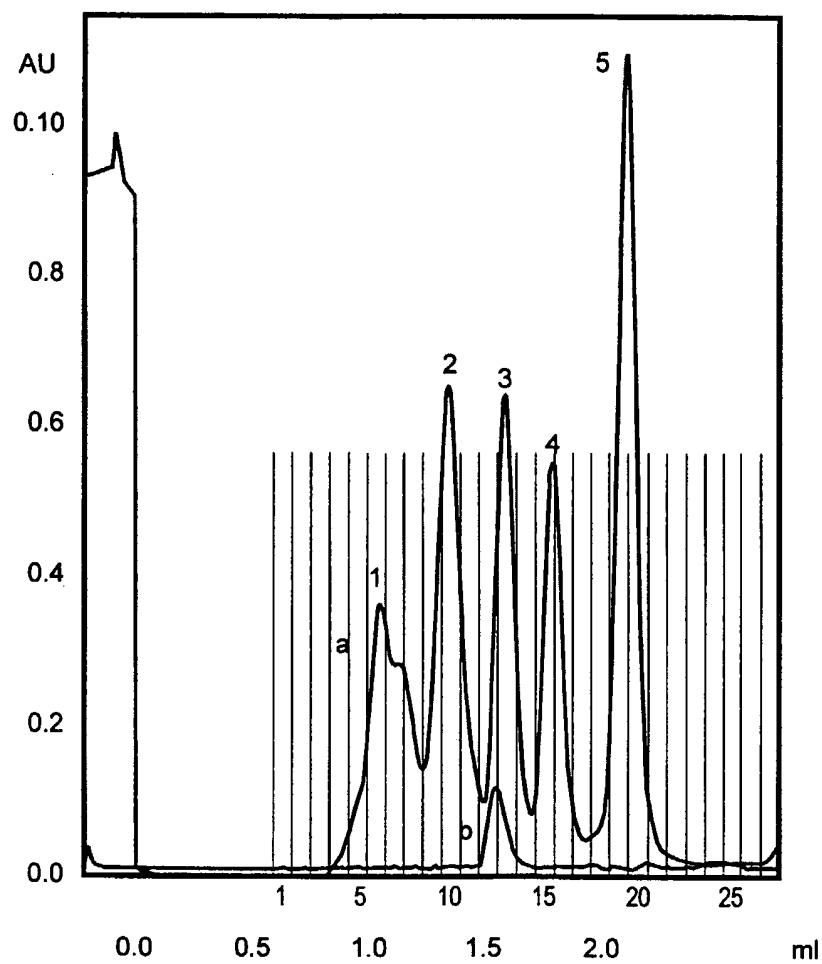


Figure 6

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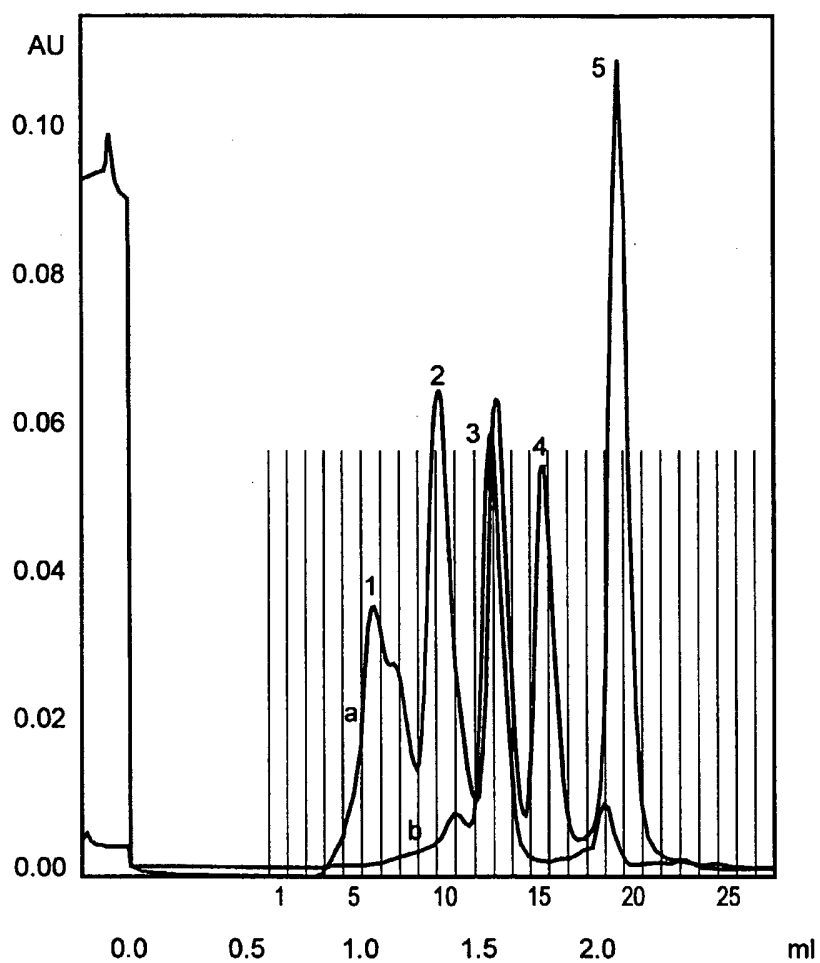


Figure 7

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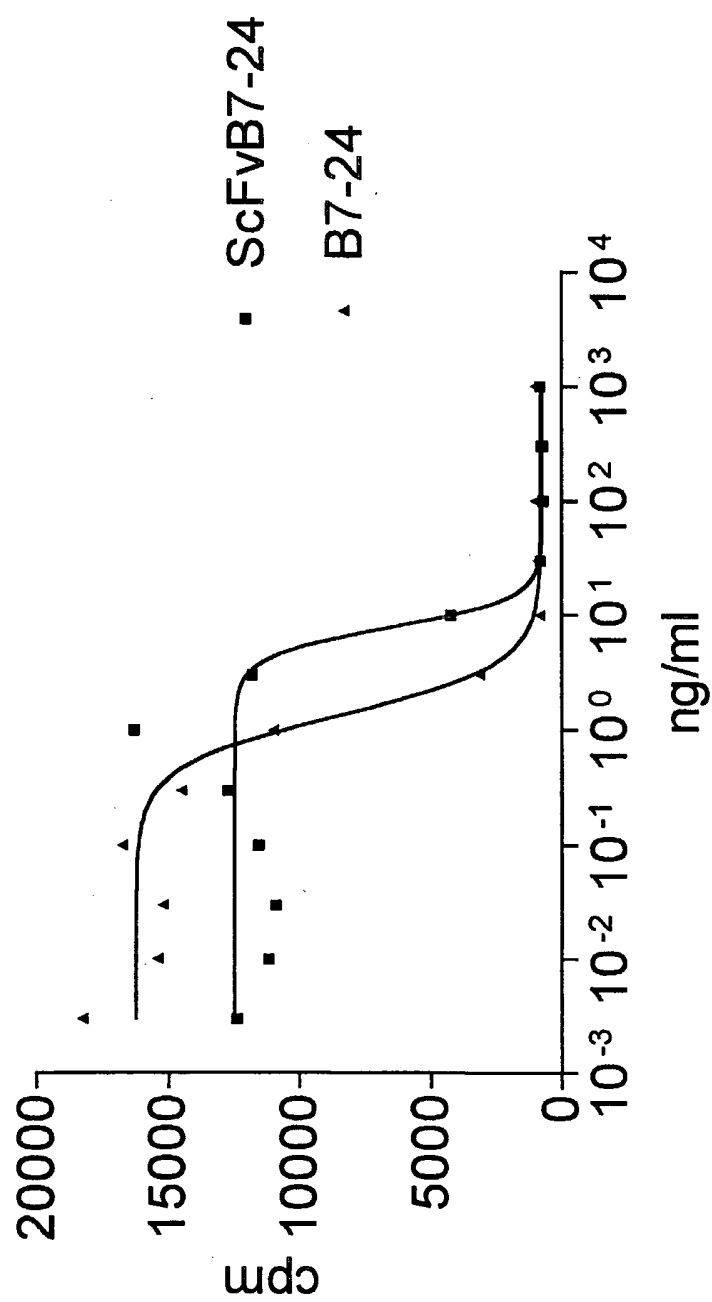


Figure 8

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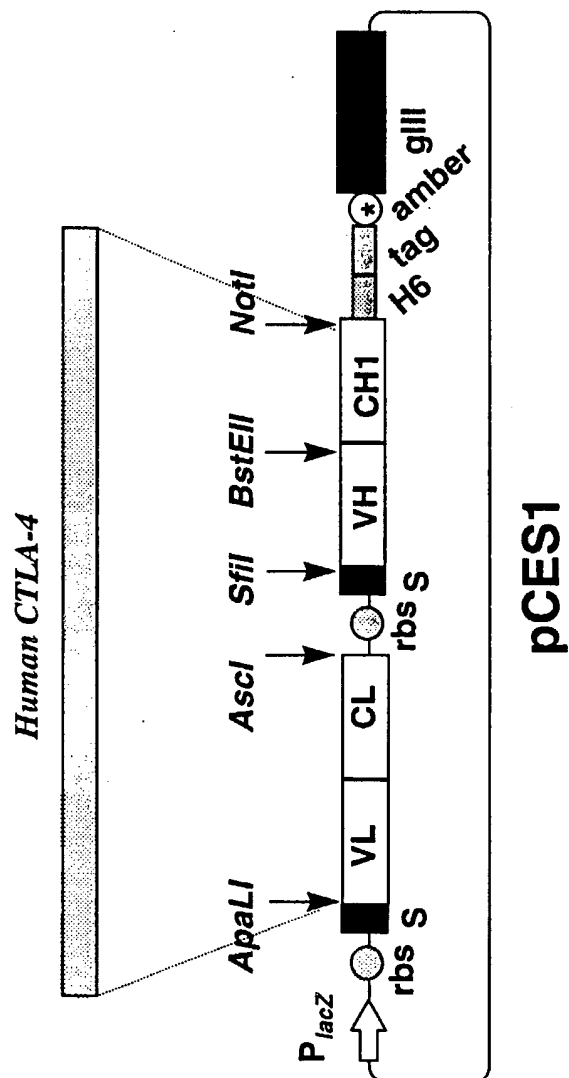
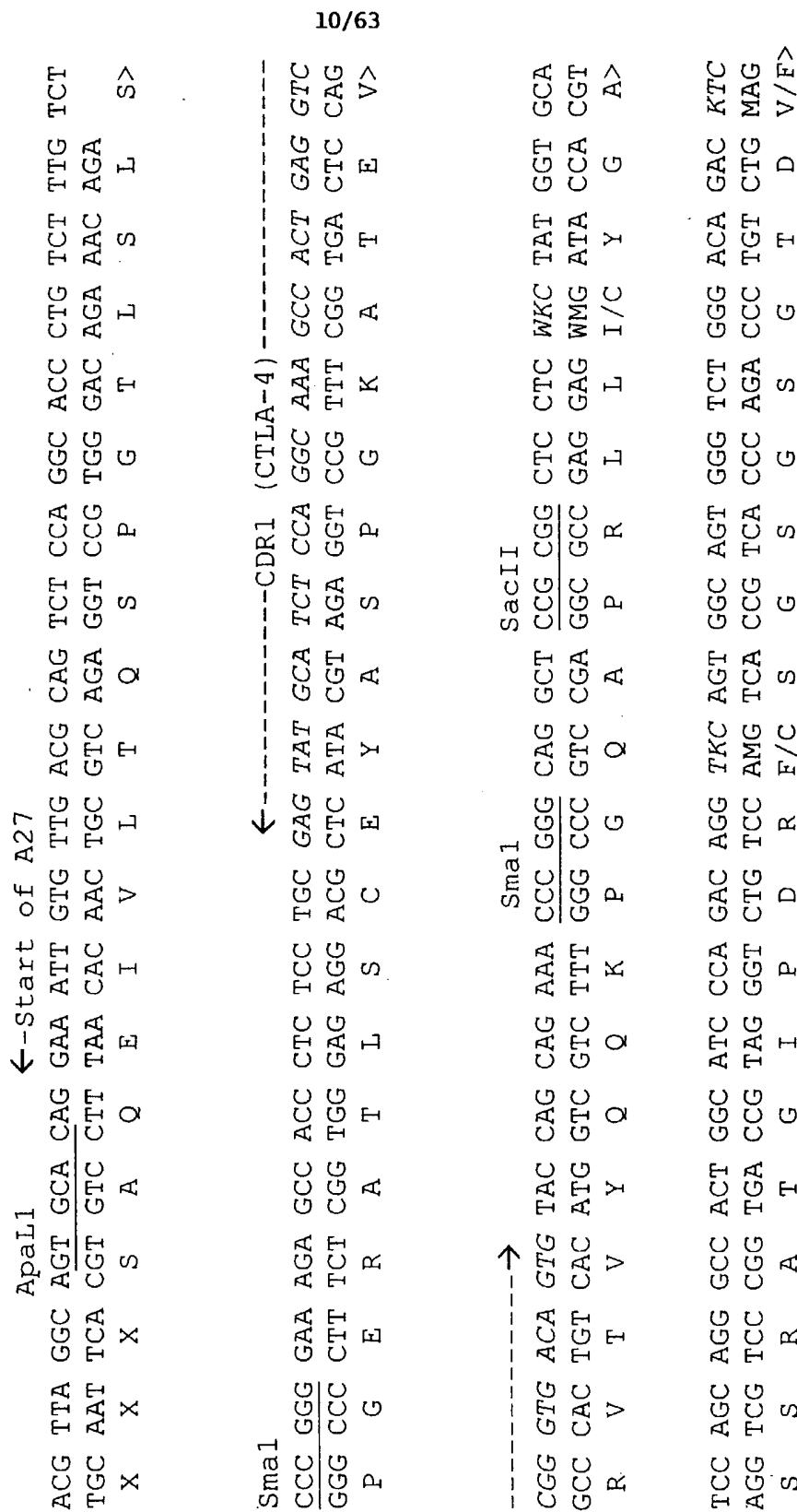


Figure 9



Figure 10



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Figure 10 cont'd 1

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      Xba1
ACT CTC ACC ATC TCT AGA CTG GAG CCT GAA GAT TTT GCA GTG TAT TAC TGT AAG GTG GAG
TGA GAG TGG TAG AGA TCT GAC CTC GGA CTT CTA AAA CGT CAC ATA ATG ACA TTC CAC CTC
T   L   T   I   S   R   L   E   P   E   D   F   A   V   Y   Y   C   K   V   E>
      ←-----

      Kpn1
---CDR 3 (CTLA-4)-----→
CTC ATG TAC CCA CCG CCA TAC TAC CTG GGC ATA GGC CAA GGT ACC AAG GTG GAA ATC AAA
GAG TAC ATG GGT GGC GGT ATG ATG GAC CCG TAT CCG GTT CCA TGG TTC CAC CTT TAG TTT
L   M   Y   P   P   P   Y   Y   L   G   I   G   Q   G   T   K   V   E   I   K>

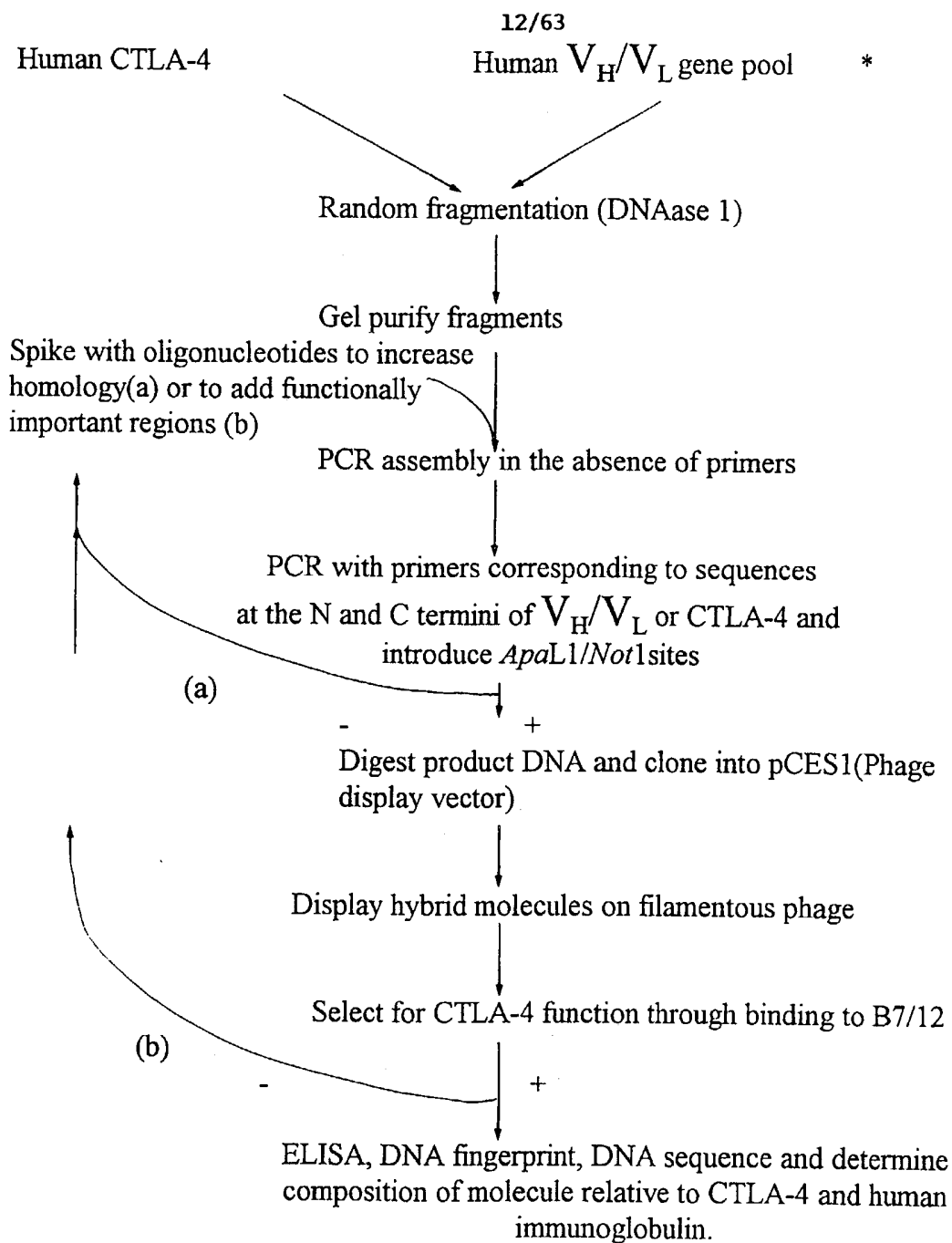
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Not1

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GCG GCC GCA ACG CCG GCG AGC
CGC CGG CGT TGC GGC CGC TCG
A A A X X X X

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\* Or a subset of  $V_H/V_L$  scaffolds which are structurally homologous to CTLA-4 as determined by structure based modeling

Figure 11

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3'-----FR3 Vklight chain-----CTLA-4 CDR3-----FR3Jk light chain-----5'

FR3 Vk light chain

E/D D FVA A/G TVY Y Y/F C  
3' CTH CTA HRV CSY HDH ATR AWR ACR

CTLA-4 CDR3 sequence

K V E L M Y P P P Y Y L G I G  
TTC CAC CTC GAG TAC ATG GGT GGC GGT ATG ATG GAC CCG TAT CCG

FR4 Jk light chain

Q G T K V E I K (SEQ ID 55)  
SBH GGG ACC AAG GTG GAA ATC AAA 5'

Figure 12

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3'-----FR3 VA light chain-----CTLA-4 CDR3-----FR3 VA light chain-----5'

FR3 VA light chain sequence

EMD D E A/S D/E Y Y/H C  
3' YHN CTR CTY MKR CTV ATR ATG ACR

CTLA-4 CDR3 sequence

K V E L M Y P P P Y Y L G I G  
TTC CAC CTC GAG TAC ATG GGT GGC GGT ATG ATG GAC CCG TAT CCG

FR4 JA light chain sequence

T G T K V T V L (SEQ ID 56)  
YSW GGG ACC AAG GTC ACC GTC CTA 5'

Figure 13

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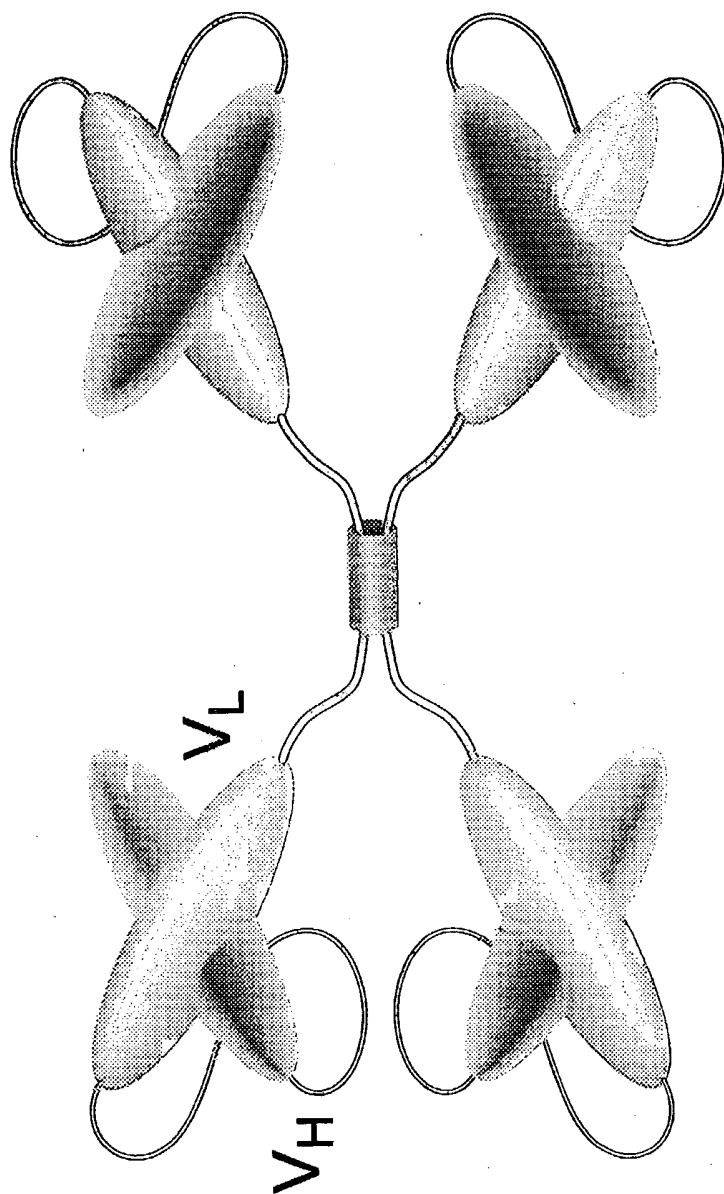


Figure 14

Figure 15

1 ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCC  
52 CAACCAGCGATGGCCCAGGTGCAGCTACAGGAGTCTGGGGGAGGCTTAGTG  
104 CAGCCTGGAGGGTCCCGGCGGCTCTCCTGTGCAGCCTCTGGATTCACTTTC  
155 AGTAGTTTTTGAATGCACTGGGTTCGT CAGGCTCCAGGGAAGGGGCTGGAA  
206 TGGGTGCGATT CATTAGTAGTGT CAGTACTCTCATCTACTATGCAGACTCA  
257 GTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTTC  
308 CTGCAAATGAACAGTCTAAGGGCAGAGGACACGGCCGTATATTACTGTGCA  
359 AGAGACGGCTGGTACTTCGATGTCTGGGGCCAAGGGACCACGGTCATCGTC  
410 TCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCG  
461 GACATCGAGCTCACTCAGTCTCCATCTTCTATGGCTGCATCTGTAGGGGAT  
512 CGGGTCACCATCACCTGCAGTGT CAGCTCAAGAATAAGTTCCAGCAATTTG  
563 CACTGGTACCAACAGAAGTCAGAACTTCCCCCAAACCCTGGATTTATGGC  
614 ACATCCAACCTGGCTTCTGGAGTCCCTTCTCGCTTCAGTGGCAGTGGATCT  
665 GGGACCGATTATACCCTCACAATCAGCAGCATGCAGCCAGAAGATGCTGCC  
716 ACTTATTACTGTCAACAGTGGAGTAGTTATCCACTCACGTTCCGGTCAGGGG  
767 ACCAAGCTGGAGCTGAAACGGACCCCGCTGGGTGATACCACTCATACCTCC  
818 GGAGGTGAACTGGAAGAGCTGTTGAAACATCTGAAAGAACTGCTGAAAGGT  
869 CCGCGGAAAGGTGAACTGGAGGAATTGCTGAAGCACCTGAAAGAGCTGTTG  
920 AAAGGTACCCCCCTAGGTGATACTACCCATAACCAGCGGTCAGGTGAAACTG  
971 CAGCAGTCTGGACCTGAGCTGGAGAAGCCTGGCGCTTCAGTGAAGATATCC  
1022 TGCAAGGCTTCTGGTTACTCATTCACTGGCCACAACATGAACTGGGTGAAG  
1073 CAGAGCAATGGAAAGAGCCTTGAGTGGATTGGAATTATTGATCCCTACTAT  
1124 GGTGGTACTAGCTACAACCCGAAGTTCGAGGGCAAGGCCACATTGACTGTA  
1175 GACAAATCCTCCAGCACAGCCTACATGCAGCTCGAGAGCCTGACATCCGAG  
1226 GACTCTGCAGTCTATTACTGTGCAAGATTCGCCTACTATGGTGACTACTAT

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Figure 15 - cont'd

1277 TATATTATGGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGT  
1328 GGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCGAG  
1379 CTCACTCAGTCTCCATCATCTCTGGCTGTGTCTGCAGGAGAAGAGGTCACT  
1430 ATGACCTGTAAGTCCAGTCAAAGTGTTTTATACAGTTCAAATCAGAAGAAC  
1481 TACTTGGCCTGGTACCAACAGAAACCAGGGCAGTCTCCTAAATTGCTGATC  
1532 TACTGGGCATCCACTAGGGAATCAGGTGTCCCTGATCGCTTCACAGGCAGT  
1583 GGATCTGGGACAGATTTTTCTCTTACCATCAGCAGTGTACAAGCTGAGGAC  
1634 CTGGCAGTTTATTACTGTCATCAATACCTCTCCTCGTGGACGTTCCGGTGGA  
1685 GGTACCAAGCTGGAAATAAAACGGCACCATCACCATCACCAC

(SEQ ID 57)



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Figure 16

1   MKYLLPTAAGLLLLAAQPAMAVQLQESGGGLVQPGGSRRLSCAASGFTFSFG  
56   MHWVRQAPGKGLEWVAFISSVSTLIYYADSVKGRFTISRDNAKNTLFLQMNSLRA  
111   EDTAVYYCARDGWYFDVWGQGTIVIVSSGGGGGGGGSDIELTQSPSSMA  
166   ASVGDRVITITCSVSSRISSSNLHWYQQKSETSPKPWIYGTSNLASGVPSRFSGSG  
221   SGTDYTLTISSMQPEDAATYYCQWSSYPPLTFGQGTKLEIKRTPPLGDTTHTSGGE  
276   LEELLKHLKELLKGPCKGELEELLKHLKELLKGTPLGDTTHTSGQVQLVQVQLQQ  
331   SGPELEKPGASVKISCKASGYSFTGHNMNWWVKQSNKGSLEWIGIIDPYYGGTSYN  
386   PKFEGKATLTVDKSSSTAYMQLESLTSEDSAVYYCARFAYYGDYYYIMDYWGQGT  
441   TVTIVSSGGGGGGGGGGSDIELTQSPSSLAVSAGEEVTMTCKSSQSVLYSSN  
496   QKNYLAWYQQKPGQSPKLLIYWASTRESGVDPDRFTGSGSGTDFSLTISSVQAEDL  
551   AVYYCHQYLSWTFGGGTKLEIKRHHHHH (SEQ ID 57)

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Figure 17

1 CAGCTACAGCAGTCTGGACCTGAGCTGGAGAAGCCTGGCGCTTCAGTGAAG  
52 ATATCCTGCAAGGCTTCTGGTTACTCATTCACTGGCCACAACATGAACTGG  
104 GTGAAGCAGAGCAATGGAAAGAGCCTTGAGTGGATTGGAATTATTGATCCC  
155 TACTATGGTGGTACTAGCTACAACCCGAAGTTCGAGGGCAAGGCCACATTG  
206 ACTGTAGACAAATCCTCCAGCACAGCCTACATGCAGCTGGAGAGCCTGACA  
257 TCCGAGGACTCTGCAGTCTATTACTGTGCAAGATTCGCCTACTATGGTGAC  
308 TACTATTATATTATGGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCC  
359 TCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAC  
410 ATCGAGCTCACTCAGTCTCCATCATCTCTGGCTGTGTCTGCAGGAGAAGAG  
461 GTCACTATGACCTGTAAGTCCAGTCAAAGTGTTTTATACAGTTCAAATCAG  
512 AAGAACTACTTGGCCTGGTACCAACAGAAACCAGGGCAGTCTCCTAAATTG  
563 CTGATCTACTGGGCATCCACTAGGGAATCAGGTGTCCCTGATCGCTTCACA  
614 GGCAGTGGATCTGGGACAGATTTTTCTCTTACCATCAGCAGTGTACAAGCT  
665 GAGGACCTGGCAGTTTATTACTGTCATCAATACCTCTCCTCGTGGACGTTT  
716 GGTGGAGGTACCAAGCTCGAGATCAAACGGACCCCGCTGGGTGATACTACT  
767 CATACTCCGGAGGTGAACTGGAAGAGCTGTTGAAACATCTGAAAGAAGT  
818 CTGAAAGGTCCGCGGAAAGGTGAACTGGAGGAATTGCTGAAGCACCTGAAA  
869 GAGCTGTTGAAAGGTACCCCCCTAGGTGATACTACCCATAACAGCGGTCTAG  
920 GTGCAACTAGTTCAGGTGCAGCTACAGGAGTCTGGGGGAGGCTTAGTGCAG  
971 CCTGGAGGGTCCCGGCGGCTCTCCTGTGCAGCCTCTGGATTCACTTTTCACT  
1022 AGTTTTTGAATGCACTGGGTTCGTCAGGCTCCAGGGAAGGGGCTGGAATGG  
1073 GTCGCATTTCATTAGTAGTGTCTAGTACTCTCATCTACTATGCAGACTCAGTG  
1124 AAGGGCCGATTACCATCTCCAGAGACAATGCCAAGAACACCCTGTTCTCTG  
1175 CAAATGAACAGTCTAAGGGCTGAGGACACGGCCGTATATTACTGTGCAAGA  
1226 GACGGCTGGTACTTCGATGTCTGGGGCCAAGGGACCACGGTCATCGTCTCC  
1277 TCAGGTGGAGGCGGTTTCAAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAC  
1328 ATCGAGCTCACTCAGTCTCCATCTTCTATGGCTGCATCTGTAGGGGATCGG  
1379 GTCACCATCACCTGCAGTGTCTAGCTCAAGAATAAGTTCAGCAATTTGCAC  
1430 TGGTACCAACAGAAGTCAGAACTTCCCCCAAACCCTGGATTTATGGCACA  
1481 TCCAACCTGGCTTCTGGAGTCCCTTCTCGCTTCAGTGGCAGTGGATCTGGG  
1532 ACCGATTATACCCCTCACAATCAGCAGCATGCAGCCAGAAGATGCTGCCACT  
1583 TATTACTGTCAACAGTGGAGTAGTTATCCACTCACGTTCCGGTCAGGGGACC  
1634 AAGCTCGAGATCAAACGGCACCATCACCATCA CCAC

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Figure 18

1 QLQSGPELEKPGASVKISCKASGYSTGHNMNWWKQNSGKSLEWIGIIDPYGG  
56 TSYNPKFEGKATLTVDKSSSTAYMQLESLTSEDSAVYICARFAYYGYIMDYW  
111 GQGTTVTVSSGGGGGGGGGGSDIELTQSPSSLAVSAGEEVTMTCKSSQSVL  
166 YSSNQKNYLAWYQQKPGQSPKLLIYWASTRESGVPRDFTGSGSGTDFSLTISVVQ  
221 AEDLAVYYCHQYLSWTFGGGTKLEIKRTPLGDTTHTSGGELEELLKHLKELLKG  
276 PRKGELEELLKHLKELLKGTPLGDTTHTSGQVQLVQVQLQESGGGLVQPGGSRRL  
331 SCAASGFTFSFGMHWVRQAPGKGLEWVAFISSVSTLIYYADSVKGRFTISRDN  
386 KNTLFLQMNSLRAEDTAVYYCARDGWYFDVWGQGTIVIVSSGGGGGGGGGGGG  
441 SDIELTQSPSSMAASVGDRTITCSVSSRISSSNLHWYQQKSETSPKPWIYGT  
496 LASGVPSRFGSGGTDYTLTISMQPEDAATYYCQWSSYPLTFGQGTKLEIKR  
551 HHHHHH (SEQ ID 59)

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Figure 19

```
1  ACCCCGCTGGGTGATACCACTCATACCTCCGGAGGTGAACTGGAAGAGCTG
52  TTGAAACATCTGAAAGAACTGCTGAAAGTCCGCGGAAAGGTGAACTGGAG
104 GAATTGCTGAAGCACCTGAAAGAGCTGTTGAAAGGTACCCCCCTAGGTGAT
155 ACTACCCATACCAGCGGT (SEQ ID 60)
```

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Figure 20

1 TPLGDTTHTSGGELEELLKHLKELLLKPRKGEELELLKHLKELLLKGTPLGDTTHTSG  
(SEQ ID 61)

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Figure 21

1 GACCTGCAGTACCACTTCGAACGTCTGGCGCGTGAAAAAACCCAGCTGATC  
52 CTGGAAACGAAGCGCTGGGTCGTAAACACCCGCCAGCTGTCTGAACAG  
(SEQ ID 62)

Figure 22

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1 DLQYHFERLAREKNQLILENEALGRNTAQLSEQ  
(SEQ ID 63)

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Figure 23

```
1      ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCC
52     CAACCAGCGATGGCCCAGGTGCAGCTACAGGAGTCTGGGGGAGGCTTAGTG
104    CAGCCTGGAGGGTCCCGGCGGCTCTCCTGTGCAGCCTCTGGATTCACTTTC
155    AGTAGTTTTTGGAAATGCACTGGGTTCGTCAGGCTCCAGGGAAGGGGCTGGAA
206    TGGGTCGCATTCATTAGTAGTGTCTAGTACTCTCATCTACTATGCAGACTCA
257    GTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTTC
308    CTGCAAATGAACAGTCTAAGGGCTGAGGACACGGCCGTATATTACTGTGCA
359    AGAGACGGCTGGTACTTCGATGTCTGGGGCCAAGGGACCACGGTCATCGTC
410    TCCTCAGGTGGAGGCGGTTTCAGACATCGAGCTCACTCAGTCTCCATCTTCT
461    ATGGCTGCATCTGTAGGGGATCGGGTCACCATCACCTGCAGTGTCTAGCTCA
512    AGAATAAGTTCCAGCAATTTGCACTGGTACCAACAGAAGTCAGAAACTTCC
563    CCCAAACCCTGGATTTATGGCACATCCAACCTGGCTTCTGGAGTCCCTTCT
614    CGCTTCAGTGGCAGTGGATCTGGGACCGATTATACCCTCACAATCAGCAGC
665    ATGCAGCCAGAAGATGCTGCCACTTATTACTGTCAACAGTGGAGTAGTTAT
716    CCACTCACGTTTCGGTCAGGGGACCAAGCTCGAGATCAAACGGCACCATCAC
767    CATCACCAC
```

(SEQ ID 65)



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Figure 24

1 MKYLLPTAAAGLLLLLAAQPAMAVQLQESGGGLVQPGGRRRLSCAASGFTFSFG  
56 MHWVRQAPGKGLEWVAFISSVSTLIYYADSVKGRFTISRDNAKNTLELQMNSLRA  
111 EDTAVYYCARDGWYFDVWGQGTIVIVSSGGGGSDIELTQSPSSMAASVGDRTIT  
166 CSVSSRISSSNLHWYQQKSETSPKRWIYGTSNLSAGVPSRFSGSGGTDYTLTIS  
221 SMQPEDAATYYCQQWSSYPLTFGQGTKLEIKRHHHHH  
(SEQ ID 65)

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Figure 25

```
1  ATGAATAACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCC
52  CAACCAGCGATGGCCCAAGGTGCAGCTACAGCAGTCTGGACCTGAGCTGGAG
104  AAGCCTGGCGCTTCAGTGAAGATATCCTGCAAGGCTTCTGGTTACTCATTC
155  ACTGGCCACAACATGAACCTGGGTGAAGCAGAGCAATGGAAGAGCCTTGAG
206  TGGATTGGAATTATTGATCCCTACTATGTTGGTACTAGCTACAACCCGAAG
257  TTCGAGGGCAAGGCCACATTGACTGTAGACAAAATCCTCCAGCACAGCCTAC
308  ATGCAGCTCGAGAGCCTGACATCCGAGGACTCTGCAGTCTATTACTGTGCA
359  AGATTGCCCTACTATGTTGACTACTATTATATATGGAATACTGGGGCCAA
410  GGGACCACGGTCACCGTCTCCTCAGGCGGTGGCGGATCTGACATCGAGCTC
461  ACTCAGTCTCCATCATCTCTGGCTGTGTCTGCAGGAGAAGAGGTCACTATG
512  ACCTGTAAGTCCAGTCAAAGTGTTTTATACAGTTCAAATCAGAAGAACTAC
563  TTGGCCTGGTACCAACAGAAACCAGGGCAGTCTCCTAAATTGCTGATCTAC
614  TGGGCATCCACTAGGGAATCAGGTGTCCCCTGATCGCTTCACAGGCAGTGGA
665  TCTGGGACAGATTTTCTCTTACCATCAGCAGTGTACAAGCTGAGGACCTG
716  GCAGTTTATTACTGTTCATCAATACCTCTCCTCGTGGACGTTTCGGTGGAGGT
767  ACCAAGCTCGAGATCAAACGGCACCATCACCATCACCAC
      (SEQ ID 66)
```

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Figure 26

1 MKYLLPTAAAGLLLLAAQPAMAQVQLQQSGPELEKPGASVKISCKASGYSFTGHN  
56 MNWVKQSNKGKSLWIGIIDPYGGTSYNPKFEGKATLTVDKSSSTAYMQLES LTS  
111 EDSAVYYCARFAYYGDYYIMDYWGQTTVTVSSGGGGSDIELTQSPSSLAVSAG  
166 EEVTMTCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIYWASTRESGVDPDRFTGS  
221 GSGTDFSLTISVQAEDLAVYYCHQYLSWTFGGG TKLEIKRHHHHHH  
(SEQ ID 67)

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Figure 27

1 ATGACCATGATTACGCCAAGCTTTGGAGCCCTTTTTTTTGGAGATTTTCAAC  
52 GTGAAAAAATTATTATTTCGCAATTCCTTTAGTTGTTCTCTTCTATGGGCCC  
104 AGCCGGCCATGGCCCAGGTGAAACTGCAGCAGTCTGGACCTGAGCTGGAGA  
155 AGCCTGGCGCTTCAGTGAAGATATCCTGCAAGGCTTCTGGTTACTCATTCA  
206 CTGGCCACAACATGAACCTGGTGAAGCAGAGCAATGGAAGAGCCTTGAGT  
257 GGATTGGAATTATTGATCCCTACTATGGTGGTACTAGCTACAACCCGAAGT  
308 TCGAGGGCAAGGCCACATTGACTGTAGACAAATCCTCCAGCACAGCCTACA  
359 TGCAGCTCGAGAGCCTGACATCCGAGGACTCTGCAGTCTATTACTGTGCAA  
410 GATTGCGCTACTATGGTGACTACTATATATATATGGACTACTGGGGCCAAG  
461 GGACCACGGTCACCGTCTCCTCAGGTGGAGGCGGTTTCAGACATCGAGCTCA  
512 CTCAGTCTCCATCTTCTATGGCTGCATCTGTAGGGGATCGGGTCACCATCA  
563 CCTGCAGTGTCAAGTCAAGAAATAAGTCCAGCAATTTGCACCTGGTACCAAC  
614 AGAAGTCAGAAACTTCCCCCAAACCCCTGGATTATGGCACATCCAACCTGG  
665 CTTCTGGAGTCCCCTTCTCGCTTCAGTGGCAGTGGATCTGGGACCGATTATA  
716 CCCTCACAATCAGCAGCATGCAGCCAGAAAGATGCTGCCACTTATTACTGTC  
767 AACAGTGGAGTAGTTATCCACTCACGTTCCGTCAGGGGACCAAGCTGGAGC  
818 TGAAACGGCACCATCACCATCACCAC (SEQ ID 68)

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Figure 28

1 MTMITPSFGAFFLEIFNVKLLFAIPLVVPFYAAQPAMAQVKLQQSGPELEKPGA  
56 SVKISCKASGYSFTGHNNMNWVKQSNKGSLEWIGIIDPYGGTSPNPKFEGKATLT  
111 VDKSSSTAYMQLESLTSEDSAVYYCARFAYYGDYYIIMDYWGQGTITVTVSS  
162 GGGGSDIELTQSPSSMAASVGDRVTITCSVSSRISSSNLHWYQQKSETSPKPWI  
216 YGTSNLASGVPSRFSGGSGTDYTLTISMQPEDAATYYCQWSSYPLTFGQGTK  
271 LELKRHHHHH  
(SEQ ID 69)

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Figure 29

```
1  ATGACCATGATTACGCCAAGCTTTGGAGCCTTTTGGAGATTTTCAAC
52  GTGAAAAAATTATTATTCGCAATTCCCTTTAGTTGTTCCCTTCTATGCGGCCC
104 AGCCGGCCATGGCCAGGTCAAACTGCAGGAGTCTGGGGAGGCTTAGTGC
155 AGCCTGGAGGGTCCCGGGCTCTCCTGTGCAGCCTCTGGATCACTTTCA
206 GTAGTTTGGAAATGCACTGGGTTCGTCAGGCTCCAGGGAAGGGCTGGAAT
257 GGGTCGCATTCAATTAGTAGTGTCACTACTCTCATCTACTATGCAGACTCAG
308 TGAAGGGCCGATTACCCATCTCCAGAGACAAATGCCAAGAACACCCCTGTTCC
359 TGCAAATGAACAGTCTAAGGGCAGAGGACACGGCCGTATATTACTGTGCAA
410 GAGACGGCTGGTACTTCGATGTCTGGGGCCAAAGGACCACCGTCATCGTCT
461 CCTCAGGTGGAGCGGTTCAGACATCGAGTCACTCAGTCTCCATCATCTC
512 TGGCTGTGTCTGCAGGAGAAGAGGTCACTATGACCTGTAAGTCCAGTCAAA
563 GTGTTTATACAGTTCAAATCAGAAGAACTACTTGGCCTGGTACCACAGA
614 AACCAGGCAGTCTCCTAAATTGCTGATCTACTGGGCATCCACTAGGGAAT
665 CAGGTGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTTCTC
716 TTACCATCAGCAGTGTAAGAAGCTGAGGACCTGGCAGTTTATTACTGTCATC
767 AATACCTCTCCTCGTGGACGTTCCGGTGGAGGTACCAAGCTGGAATAAAAC
818 GG (SEQ ID 70)
```

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Figure 30

1 MTMITPSFGAFFLEIFENVKKLLFAIPLVVPFYAAQPAMAQVKLQESGGGLVQPGG  
56 SRRLSCAASGFTFSFGMHWRQAPGKGLEWVAFISSVSTLIYYADSVKGRFTIS  
111 RDNAKNTLFLQMNSLRAEDTAVYYCARDGWYFDVWGQGTIVSSGGGSD  
162 IELTQSPSSLAVSAGEEVTMTCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIY  
216 WASTRESGVDRFTGSGGTDFTLTSSVQAEDLAVYYCHQYLSSTFTGGGKLE  
271 IKR  
(SEQ ID 71)

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Figure 31

```
1  ATGAAATACCTATTGCCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCC
52  CAACCAGCGATGGCCCAAGGTGCAGCTACAGGAGTCTGGGGAGGCTTAGTG
104 CAGCCTGGAGGGTCCCGGGCTCTCCTGTGCAGCCTCTGGATTCACTTTC
155 AGTAGTTTGGAAATGCACTGGGTTTCGTCAAGGCTCCAGGGAAGGGCTGGAA
206 TGGGTCGCATTTCATTAGTAGTGTCACTACTCTCATCTACTATGCAGACTCA
257 GTGAAGGGCCGATTCAACCATCTCCAGAGACAAATGCCAAGAACAACCCCTGTTTC
308 CTGCAAAATGAACAGTCTAAGGGCTGAGGACACGGCCGTATATTAATGTGCA
359 AGAGACGGCTGGTACTTCGATGTCTGGGCCAAGGAGACCACGGTCATCGTC
410 TCCTCAGACATCGAGCTCACTCAGTCTCCATCTTCTATGGCTGCATCTGTA
461 GGGGATCGGGTCACCATCACCTGCAGTGTCAAGTCAAGAAATAAGTTCCAGC
512 AATTGCACTGGTACCACAGAAAGTCAGAAACTTCCCCAAACCCCTGGATT
563 TATGGCACATCCAAACCTGGCTTCTGGAGTCCCCTTCTCGCTTCAGTGGCAGT
614 GGATCTGGGACCGATTATACCCCTCACAATCAGCAGCATGCAGCCAGAAGAT
665 GCTGCCACTTATTACTGTCAACAGTGGAGTAGTTATCCACTCACGTTCCGGT
716 CAGGGGACCAAGCTCGAGATCAAACGGCACCACATCACCATCACCCAC
      (SEQ ID 72)
```



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Figure 32

1 MKYLLPTAAAGLLLLAAQPAMAQVQLQESGGGLVQPGGSRRLSCAASGFTFSFG  
56 MHWVRQAPGKGLEWVAFISSVSTLIYYADSVKGRFTISRDNAKNTLELQMNSLRA  
111 EDTAVYYCARDGWYFDVWGQGTIVIVSSDIELTQSPSSMAASVGDRTITCSVSS  
166 RISSNLHWYQQKSETSPKPIYGTSLASGVPSRFSGSGGTDYTLTISSMQPE  
221 DAATYYCQQWSSYPPLTFGQGTKLEIKRHHHHH  
(SEQ ID 73)

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Figure 33

1 ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCC  
52 CAACCCAGCGATGGCCAGGTGCAGCTACAGCAGTCTGGACCTGAGCTGGAG  
104 AAGCCTGGCGCTTCAGTGAAGATATCCTGCAAGGCTTCTGGTTACTCATTC  
155 ACTGGCCACAACATGAACTGGGTGAAGCAGAGCAATGGAAGAGCCTTGAG  
206 TGGATTGGAAATTATTGATCCCTACTATGGTGGTACTAGCTACAACCCGAAG  
257 TTCGAGGGCAAGGCCACATTGACTGTAGACAAATCCTCCAGCACAGCCTAC  
308 ATGCAGCTCGAGAGCCCTGACATCCGAGGACTCTGCAGTCTATTACTGTGCA  
359 AGATTCCGCTACTATGGTGACTACTATTATTATGGACTACTGGGCCAA  
410 GGGACCACGGTCACCGTCTCCTCAGACATCGAGCTCACTCAGTCTCCATCA  
461 TCTCTGGCTGTGTCTGCAGGAGAAGAGGTCACTATGACCTGTAAGTCCAGT  
512 CAAAGTGTTTTATACAGTTCAAATCAGAAGAACTACTTGGCCTGGTACCAA  
563 CAGAAACCAGGGCAGTCTCCTAAATTGCTGATCTACTGGGCATCCACTAGG  
614 GAATCAGGTGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTT  
665 TCTCTTACCATCAGCAGTGTACAAGCTGAGGACCTGGCAGTTTATTACTGT  
716 CATCAATACCTCTCCTCGTGGACGTTCCGTGGAGGTACCAAGCTCGAGATC  
767 AAACGGCACCATCACCATCACCAC

(SEQ ID 74)

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Figure 34

1 MKYLLPTAAAGLLLLAAQPAMAQVQLQQSGPELEKPGASVKISCKASGYSTGHN  
56 MNWVKQSNKGKSLIEWIGIIDPYGGTSYNPKFEGKATLTVDKSSSTAYMQLES LTS  
111 EDSAVYYCARFAYYGDYYIMDYWGQGT TTVTVSSDIELTQSPSSLA VSAGEEVTM  
166 TCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIYWASTRESGV PDRFTGSGSGTD  
221 FSLTISSVQAEDLAVYYCHQYLSSWTFGGGTKLEIKRHHHHH  
(SEQ ID 75)

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Figure 35

1 ATGACCATGATTACGCCAAGCTTTGGAGCCTTTTTTTTGAGATTTTCAAC  
52 GTGAAAAAATTATTATTTCGCAATTCCTTTAGTTGTTCTCTTCTATGCGGCCCC  
104 AGCCGGCCATGGCCAGGTGAAACTGCAGCAGTCTGGACCTGAGCTGGAGA  
155 AGCCTGGCGCTTCAGTGAAGATATCCTGCAAGGCTTCTGGTACTCATTTCA  
206 CTGGCCACAACATGAAC TGGGTGAAGCAGAGCAATGGAAGAGCCCTTGAGT  
257 GGATTGGAATTATTGATCCCTACTATGGTGGTACTAGCTACAACCCGGAAGT  
308 TCGAGGGCAAGGCCACATGACTGTAGACAAATCCTCCAGCACAGCCTACA  
359 TGCAGCTCGAGAGCCTGACATCCGAGGACTCTGCAGTCTATTACTGTGCAA  
410 GATTGCGCTACTATGGTGACTACTATTATATGGACTACTGGGGCCCAAG  
461 GGACCACGGTCACCGTCTCCTCAGACATCGAGCTCACTCAGTCTCCATCTTT  
512 CTATGGCTGCATCTGTAGGGGATCGGGTCACCATCACCTGCAGTGTGAGCT  
563 CAAGAAATAAGTTCAGCAATTTCGACTGGTACCAACAGAAAGTCAGAAACTT  
614 CCCCCAAACCCTGGATTTATGGCACATCCAACTGGCTTCTGGAGTCCCTT  
665 CTCGCTTCAGTGGCAGTGGATCTGGGACCGATTATACCTCACAAATCAGCA  
716 GCATGCAGCCAGAAGATGCTGCCACTTATTACTGTCAACAGTGGAGTAGTT  
767 ATCCACTCACGTTCCGTCAGGGGACCAAGCTGGAGCTGAAACGGCACCATC  
818 ACCATCACCAC (SEQ ID 76)

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Figure 36

1 MTMITPSFGAFFLEIFNVKLLLEAIPLVVPFYAAQPAMAQVKLQQSGPELEKPGA  
56 SVKISCKASGYSFTGHNNMNWVKQSNKGSLEWIGIIDPYYGTSYNPKFEGKATLT  
111 VDKSSSTAYMQLESLTSEDSAVYYCAREFAYYGDYYYIMDYWGQGTTVTVSS  
162 DIELTQSPSSMAASVGDRVTITCSVSSRISSSNLHWYQQKSETSPKPWIYGTSN  
216 LASGVPSRFSGSGTDYTLTISSMQPEDAATYYCQWSSYPPLTFGQGTKLELKR  
271 HHHHHH  
(SEQ ID 77)

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Figure 37

```
1  ATGACCATGATTACGCCAAGCTTTGGAGCCTTTTGGAGATTTTCAAC
52  GTGAAAAAATATTATTGCAATTCCTTTAGTTGTTCTTCTATGCGGCCCC
104  AGCCGGCCATGGCCCAAGGTCAAACCTGCAGGAGTCTGGGGAGGCTTAGTGC
155  AGCCTGGAGGGTCCCGGGGCTCTCCTGTGCAGCCTCTGGATTCACCTTCA
206  GTAGTTTGGAAATGCACTGGGTTTCGTCAAGCTCCAGGGAAGGGGCTGGAAT
257  GGGTCGCATTCAATTAGTAGTGTCACTACTCTCATCTACTATGCAGACTCAG
308  TGAAGGGCCGATTACCATCTCCAGAGACAAATGCCAAGAACACCCTGTTCC
359  TGCAAAATGAACAGTCTAAGGGCAGAGGACACGGCCGTATATTACTGTGCAA
410  GAGACGGCTGGTACTTCGATGTCTGGGGCCAAAGGACCCACGGTCATCGTCT
461  CCTCAGACATCGAGCTCACTCAGTCTCCATCATCTCTGGCTGTGTCTGCAG
512  GAGAAAGAGTCACTATGACCTGTAAGTCCAGTCAAAGTGTTTTATACAGTT
563  CAAATCAGAAAGAACTACTTGGCCCTGGTACCAACAGAAACCAGGGCAGTCTC
614  CTAATTGCTGATCTACTGGGCATCCACTAGGGAATCAGGTGTCCCTGATC
665  GCTTCACAGGCAGTGGATCTGGGACAGATTTTCTCTTACCATCAGCAGTG
716  TACAAAGCTGAGGACCTGGCAGTTTATTACTGTCAATCAATACCTCTCCTCGT
767  GGACGTTCCGTGGAGGTACCAAGCTGGAAATAAAACGG
      (SEQ ID 78)
```

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Figure 38

1 MTMITPSFGAFFLEIFNVKLLFAIPLVVPFYAAQPAMAQVKLQESGGGLVQPGG  
56 SRRLSCAASGTFSSFGMHWRQAPGKGLEWVAFISSVSTLIYYADSVKGRFTIS  
111 RDNAKNTLFLQMNSLRAEDTAVYYCARDGWYFDVWGQGTIVIVSSDIELTQ  
162 SPSSLAVSAGEEVTMTCKSSQSVLYSSNQKNYLAWYQQKPGQSPKLLIYWASTR  
216 ESGVPDRFTGSGGTDFTSLTISSVQAEDLAVYYCHQYLSWTFGGGKLEIKR  
(SEQ ID 79)

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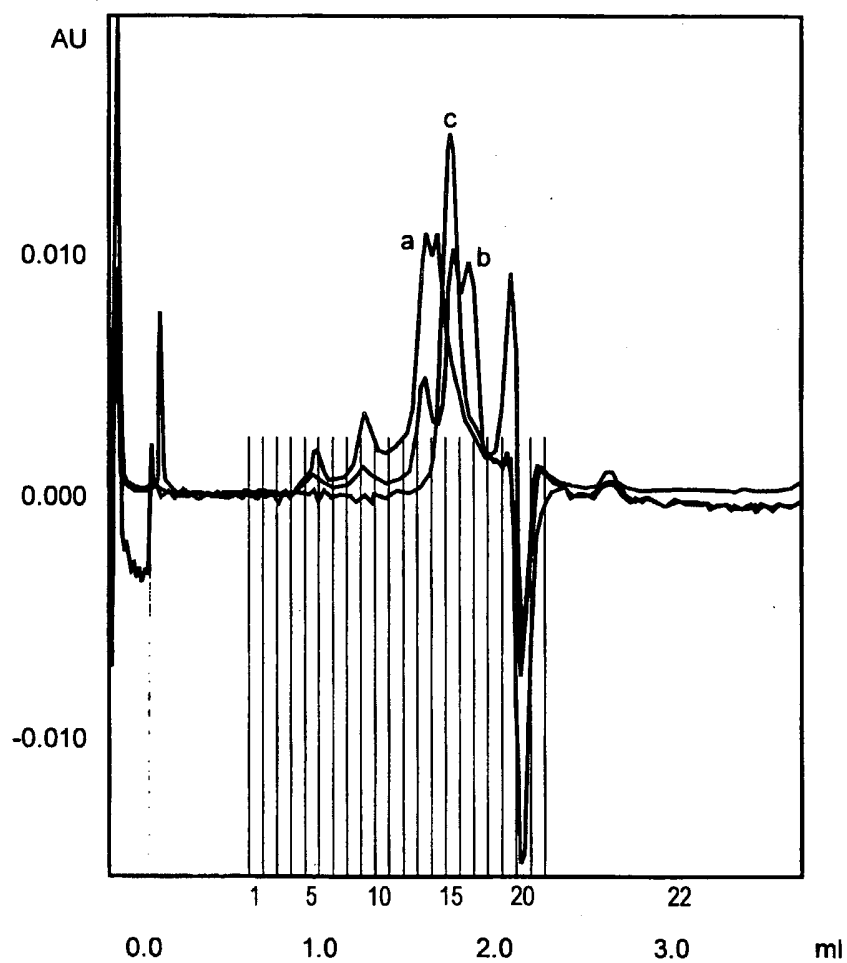


Figure 39



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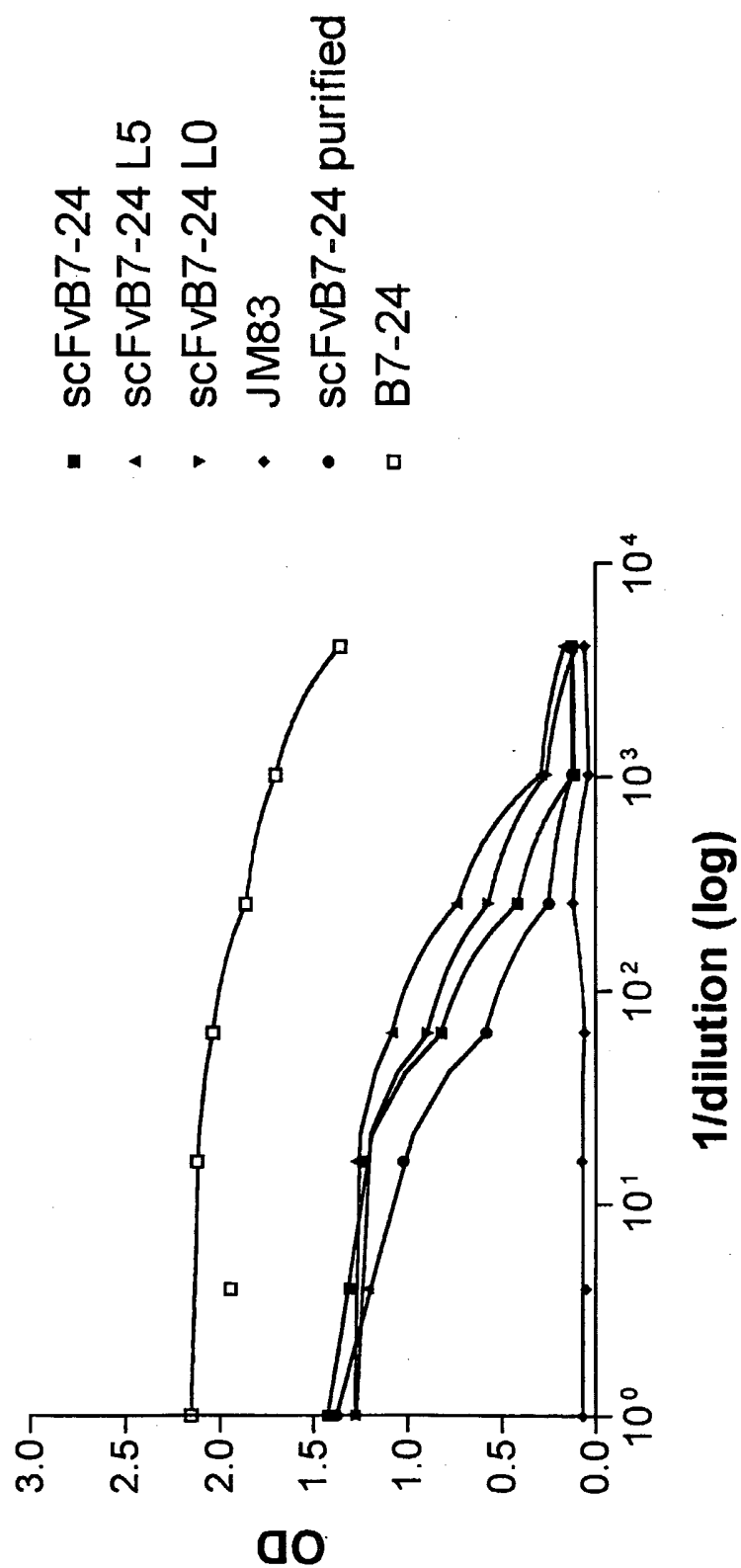
**B7.1ED fusion protein**

Figure 40

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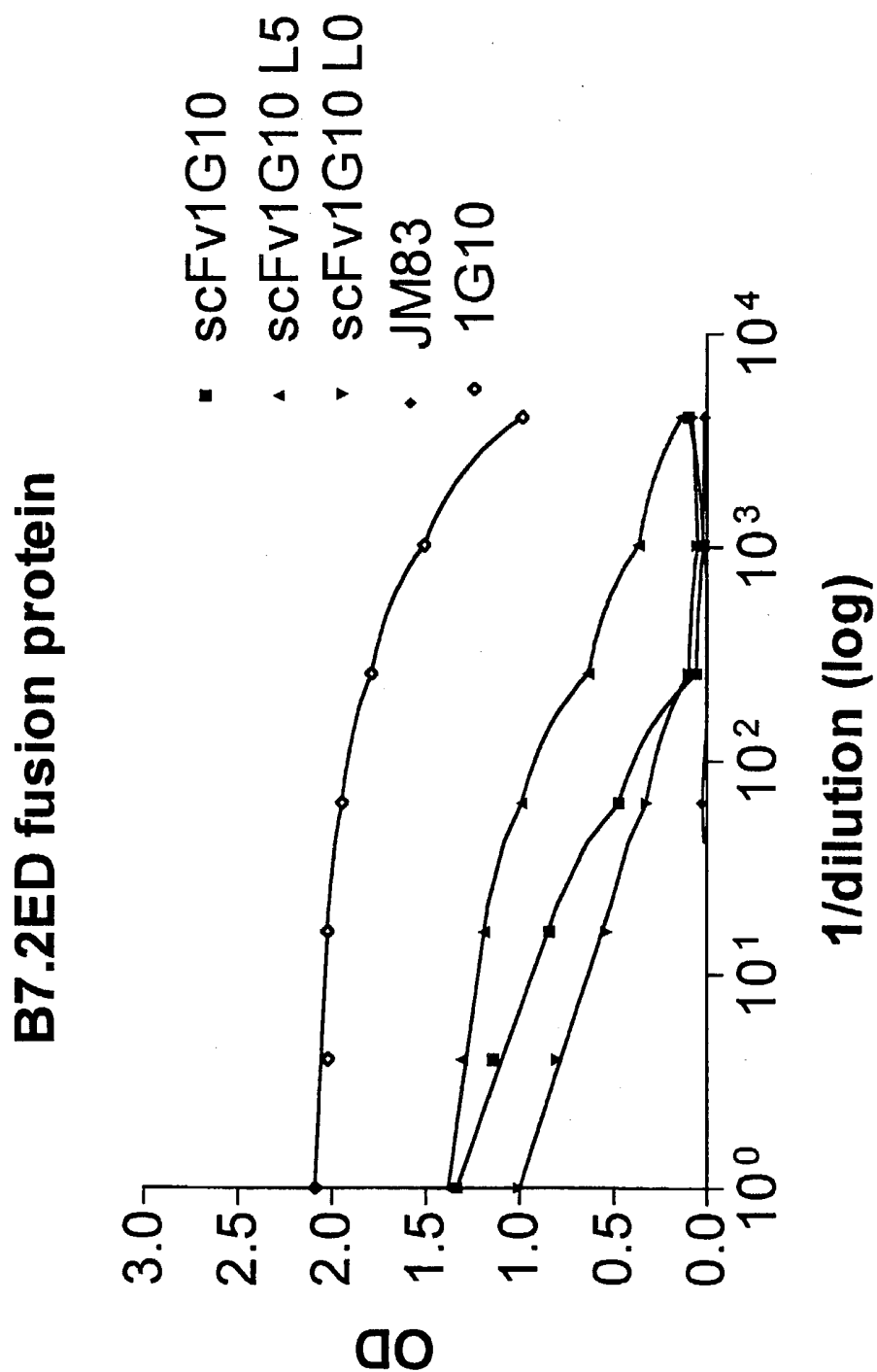
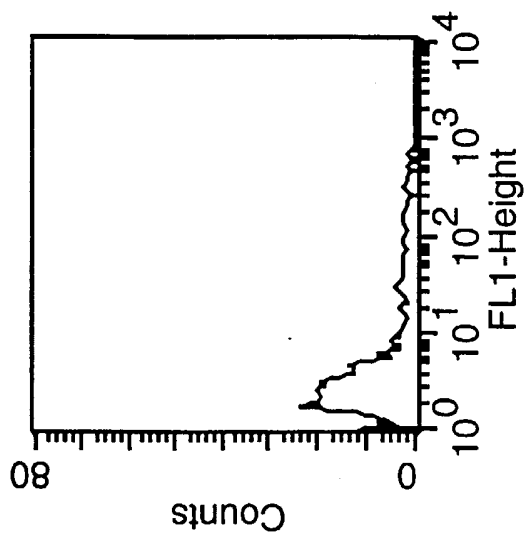


Figure 41

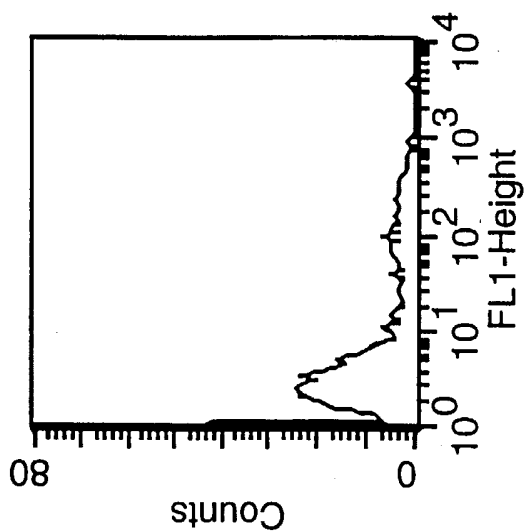
44/63

RPMI 8866



aHis + aMo-FITC

3T6/CD32/B7.1

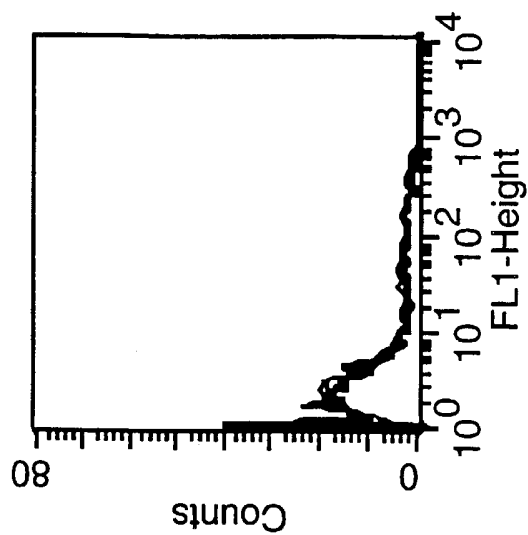


aHis + aMo-FITC

Figure 42 a

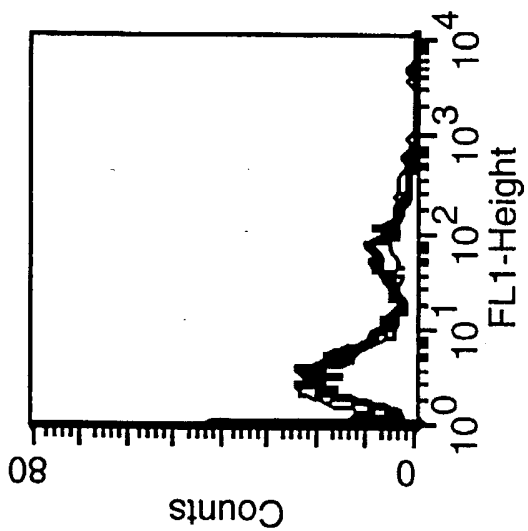
45/63

RPMI 8866



JM 83

3T6/CD32/B7.1



JM 83

Figure 42 a - cont'd

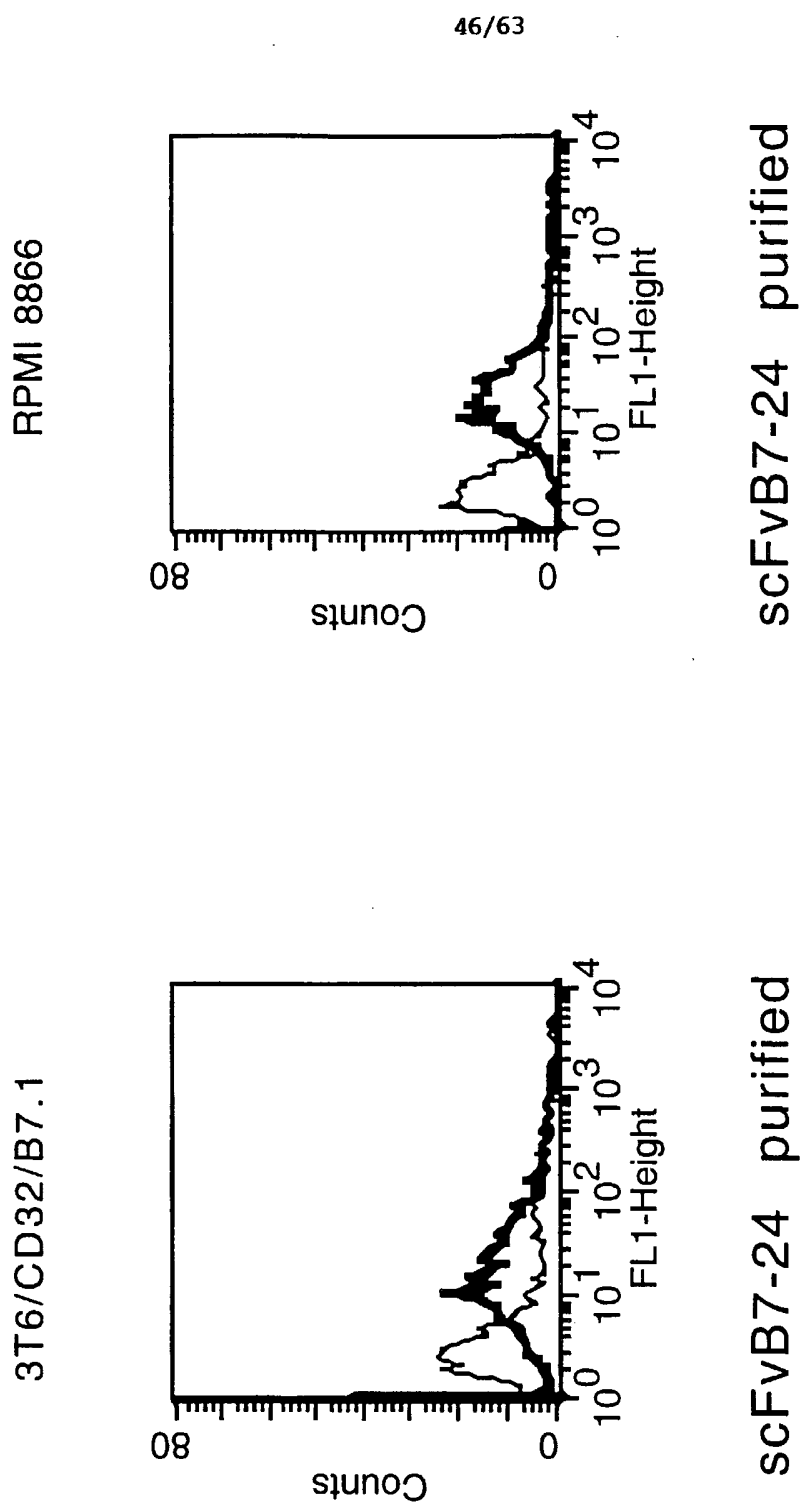
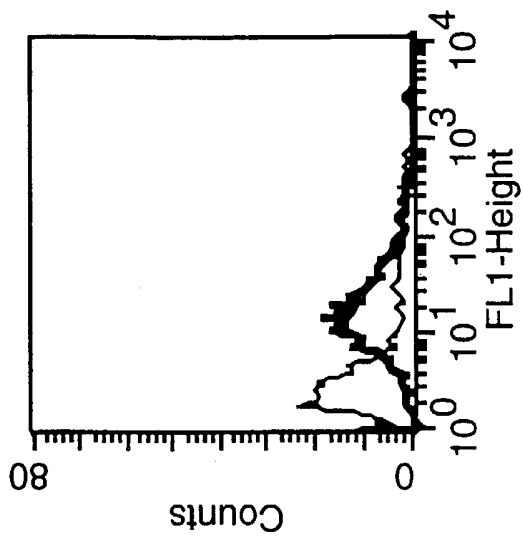


Figure 42 b

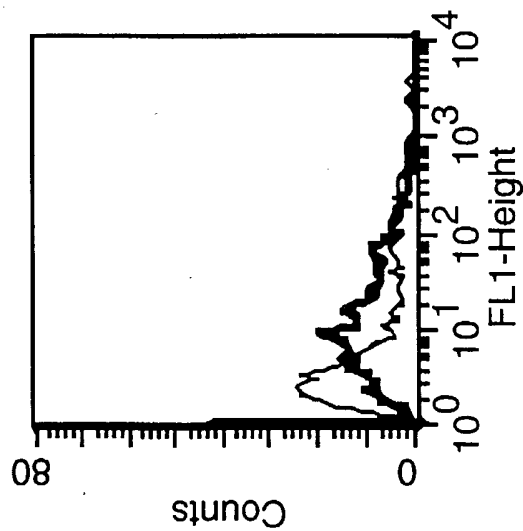
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RPMI 8866



crude scFvB7-24

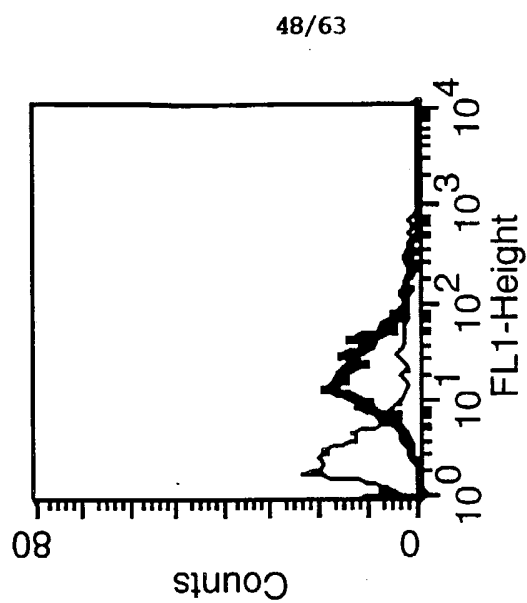
3T6/CD32/B7.1



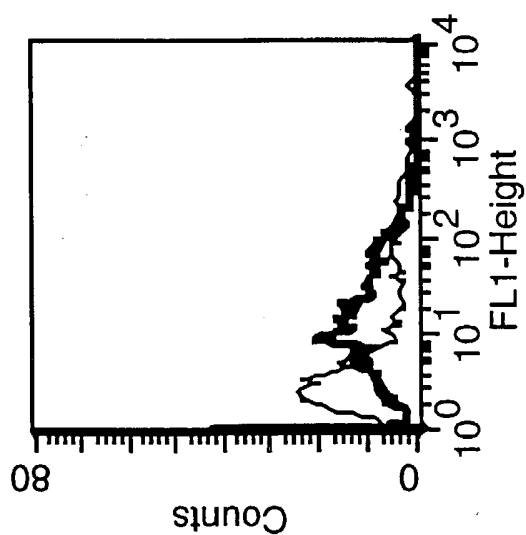
crude scFvB7-24

Figure 42 b - cont'd

RPMI 8866



3T6/CD32/B7.1



crude scFvB7-24 L5

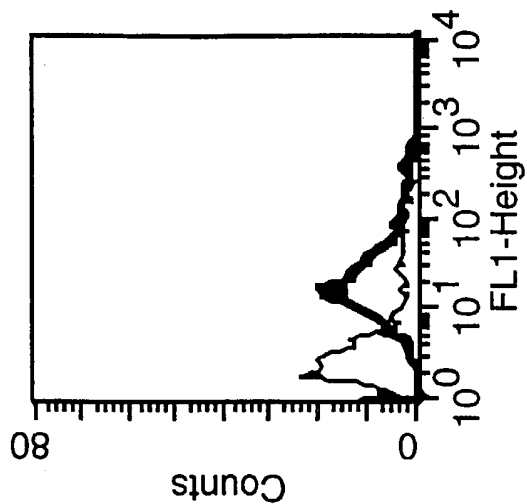
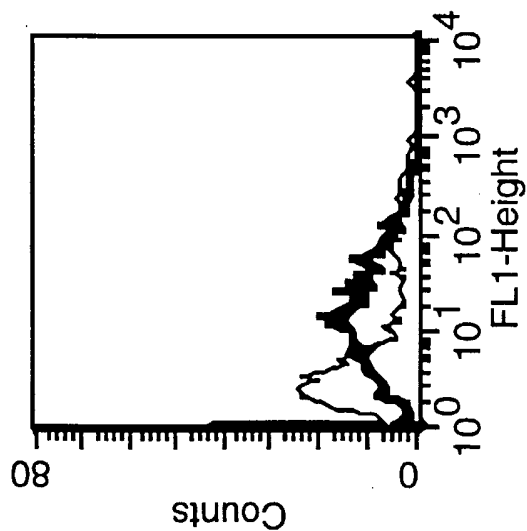
crude scFvB7-24 L5

Figure 42 c

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3T6/CD32/B7.1

RPMI 8866



crude scFvB7-24 L0

crude scFvB7-24 L0

Figure 42 c - cont'd



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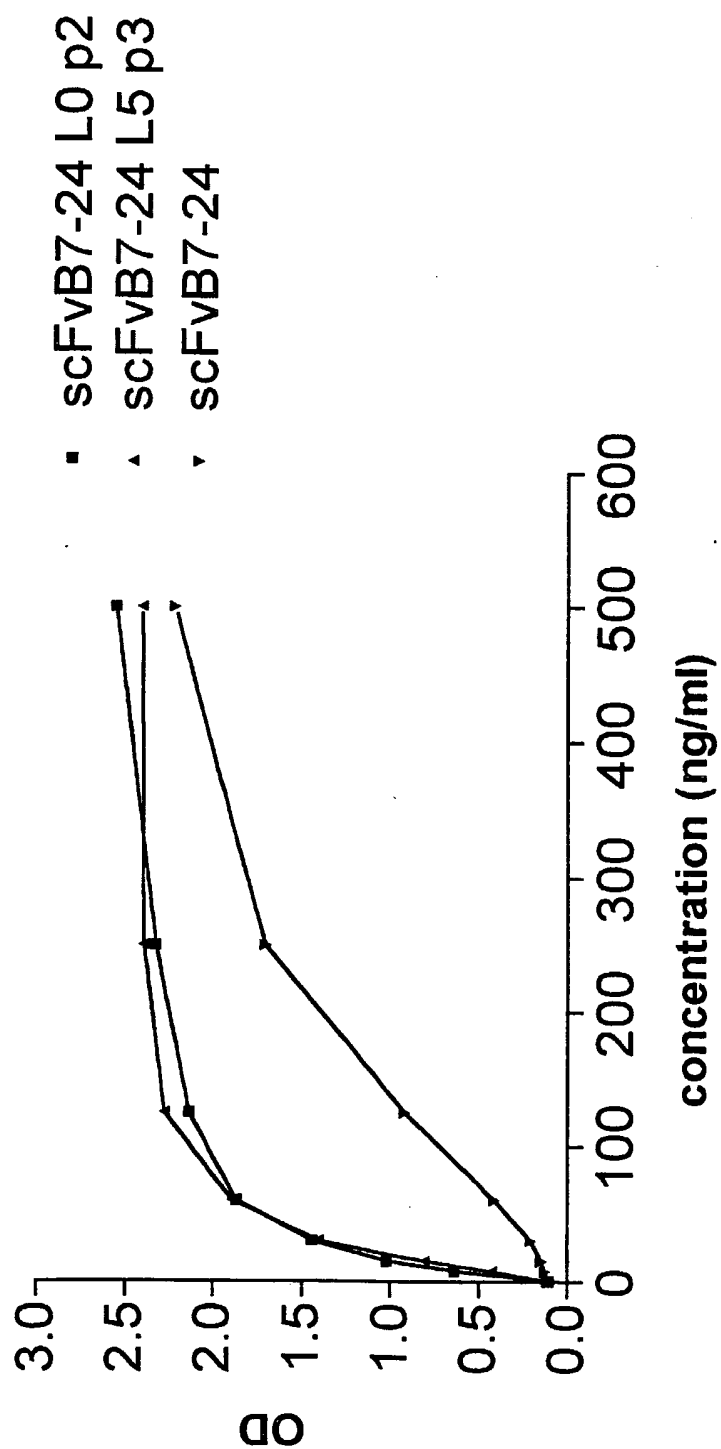
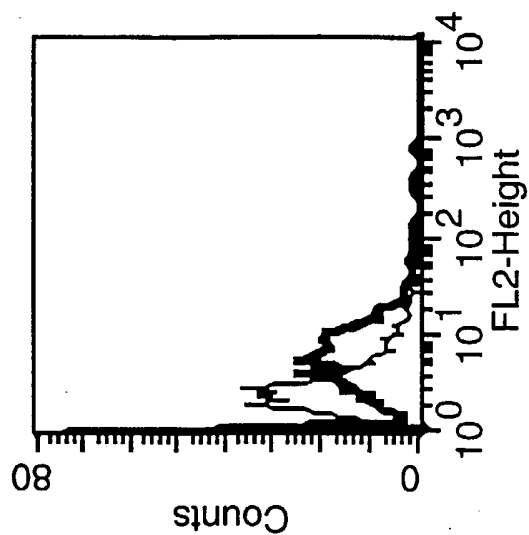


Figure 43

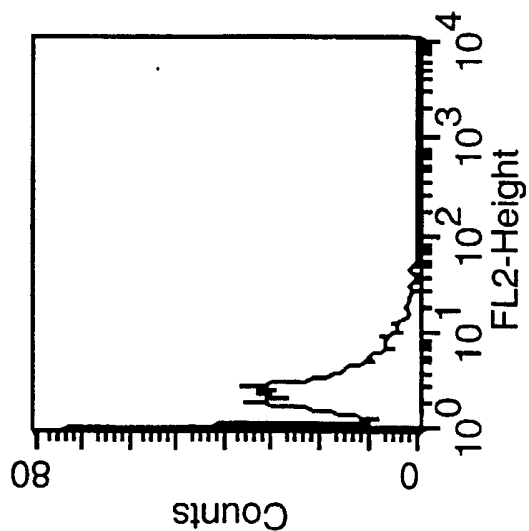
51/63

RPMI 8866



scFvB7-24

RPMI 8866

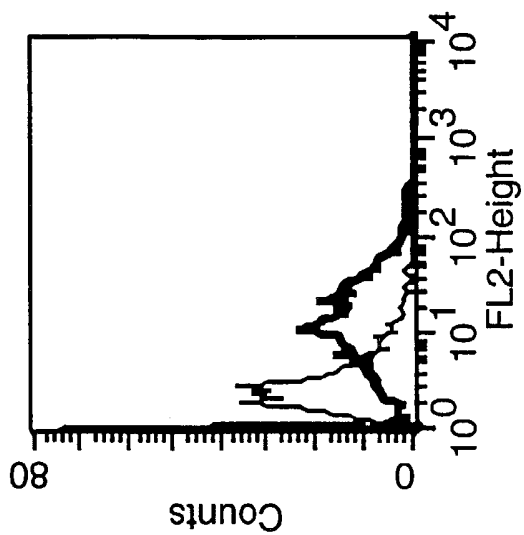


aHis-aMo.bio-strep.PE

Figure 44 a

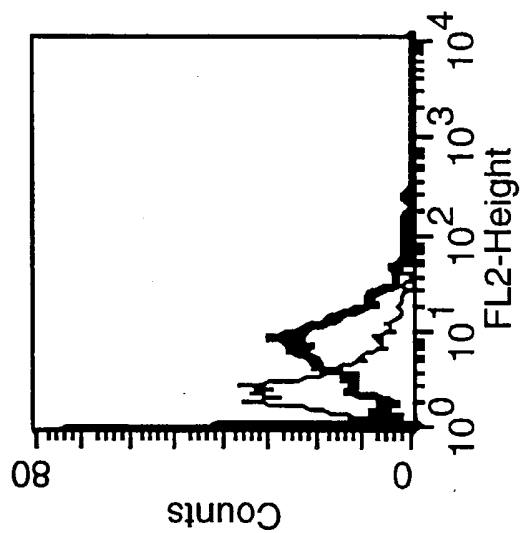
52/63

RPMI 8866



ScFvB7-24 L5 p2

RPMI 8866

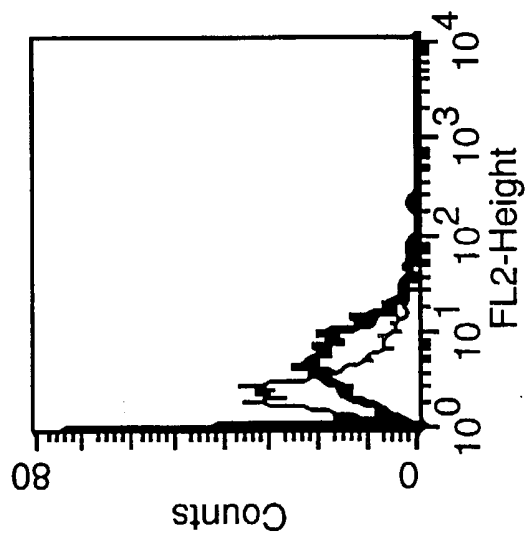


ScFvB7-24 L0 p2

Figure 44 b

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RPMI 8866



ScFvB7-24 L5 p3

Figure 44 b - cont'd

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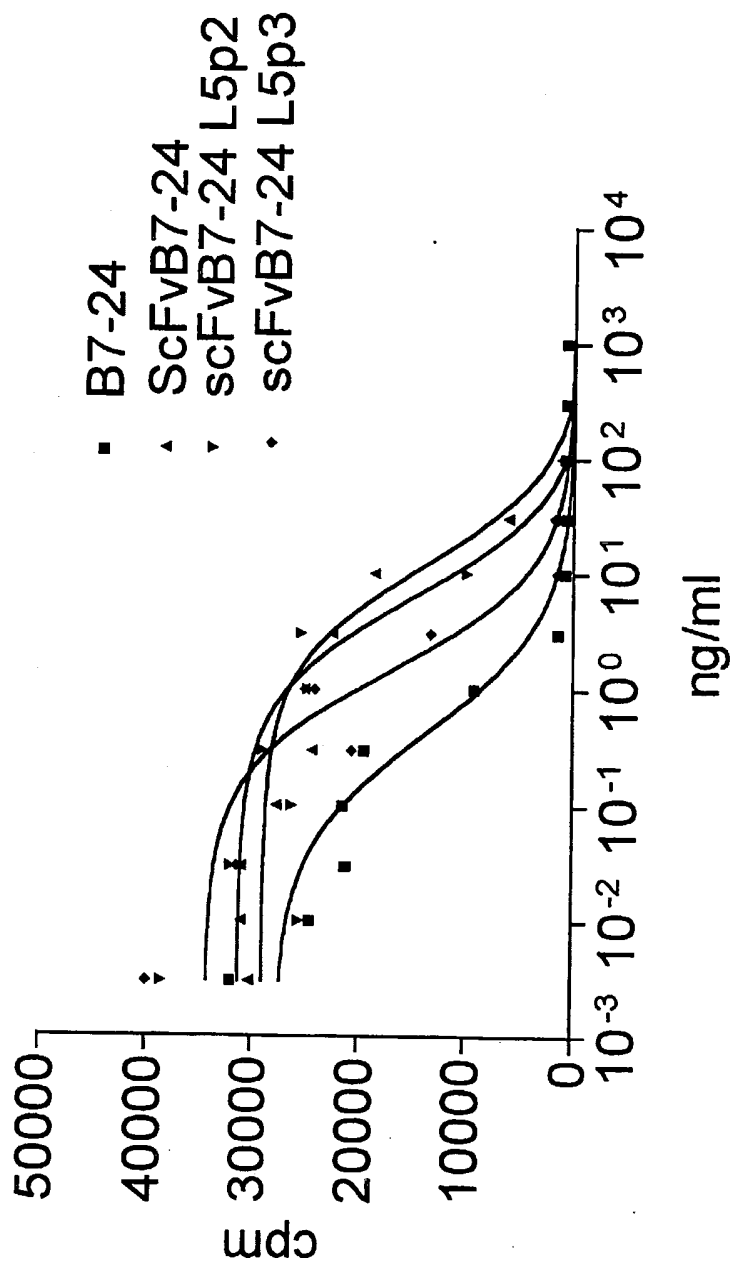


Figure 45

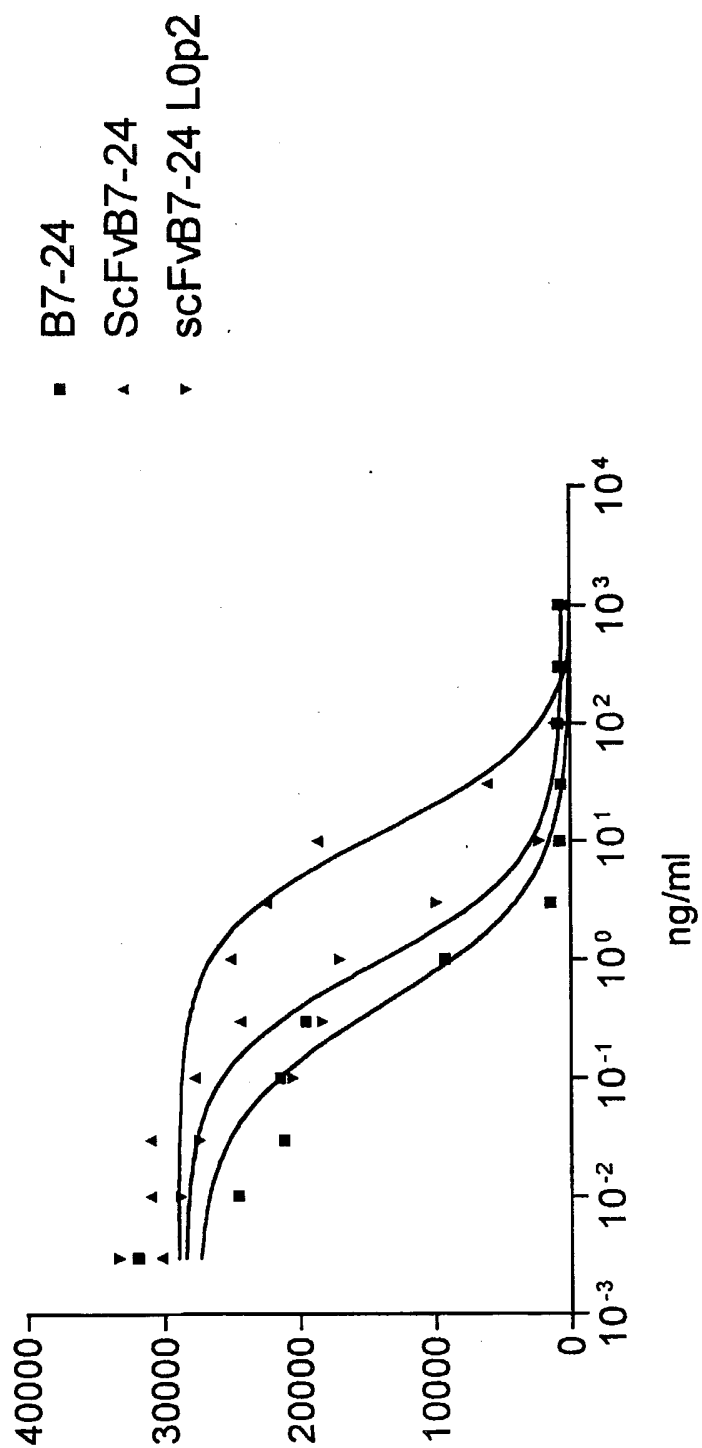


Figure 46

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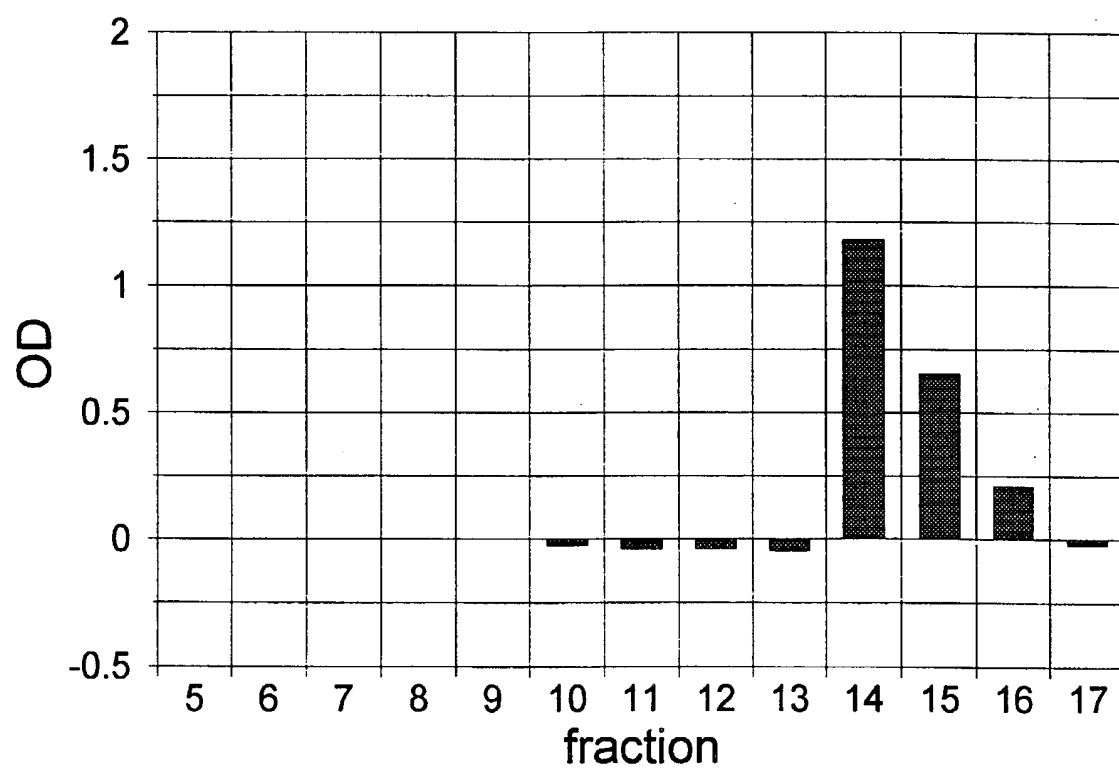


Figure 47

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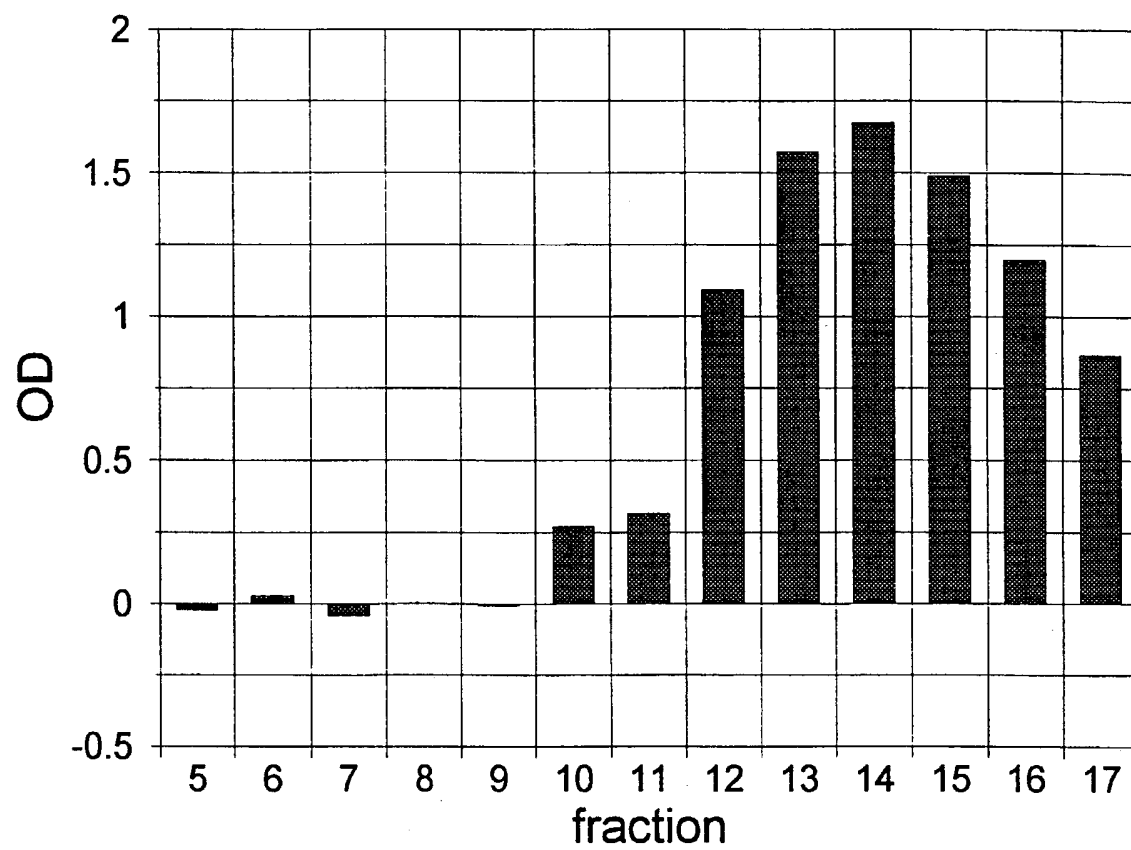


Figure 47 - cont'd 1



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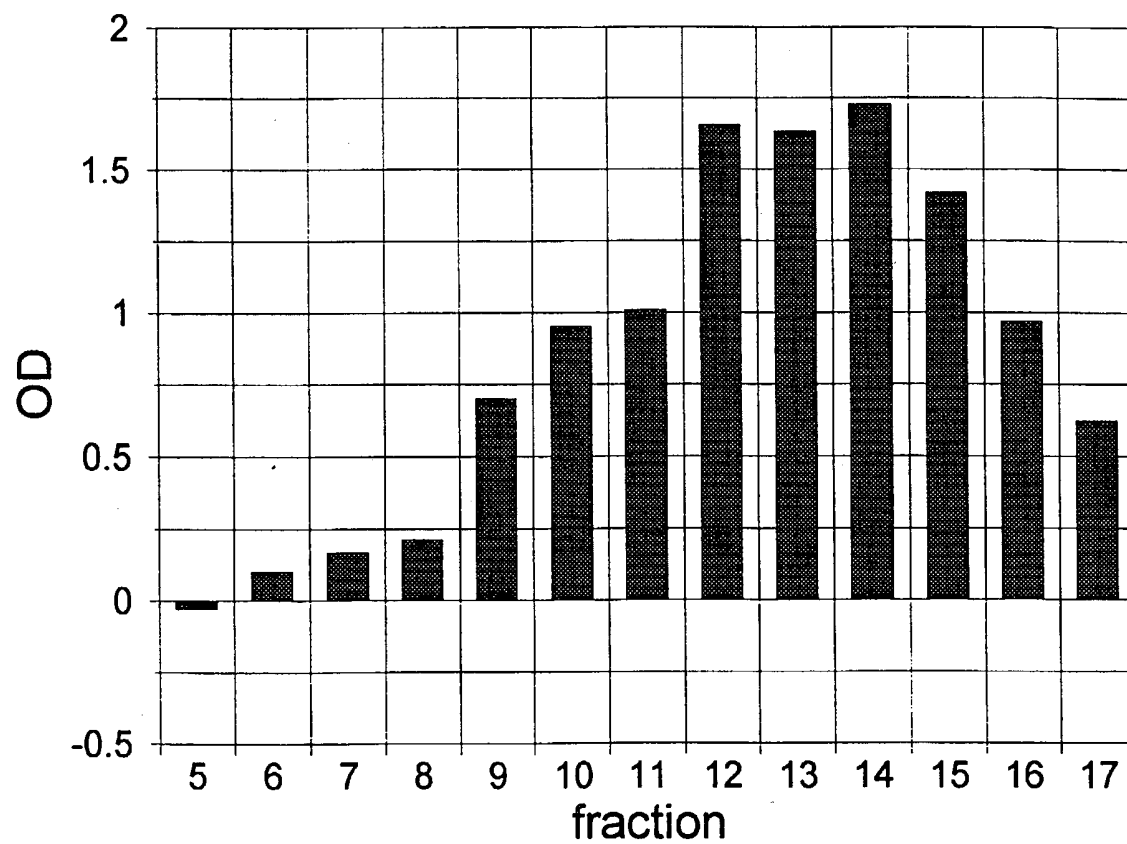


Figure 47 - cont'd 2

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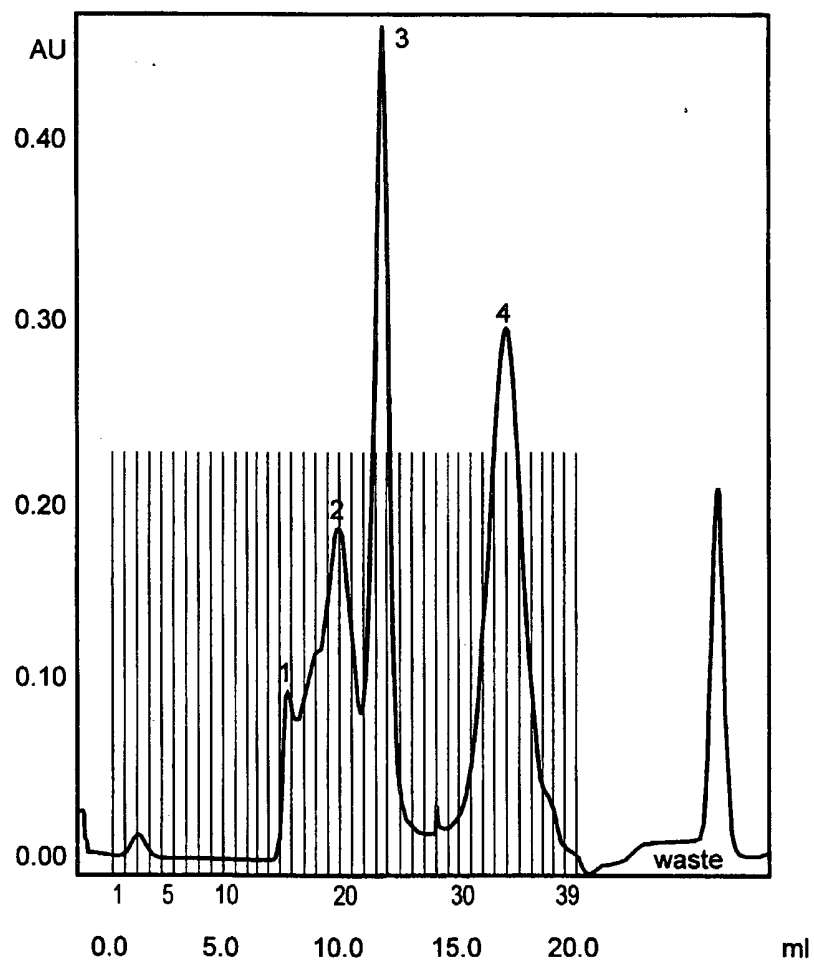
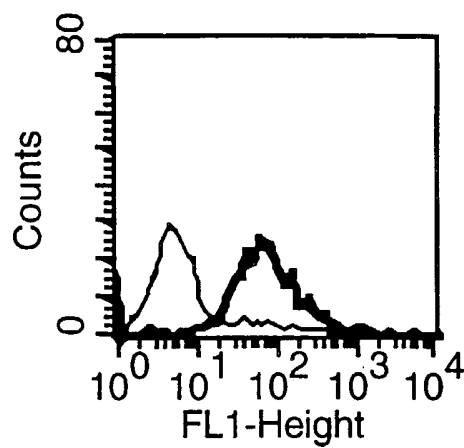


Figure 48

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**FRACTION 19**

3T6/CD32/B7.1



3T6/CD32/B7.2

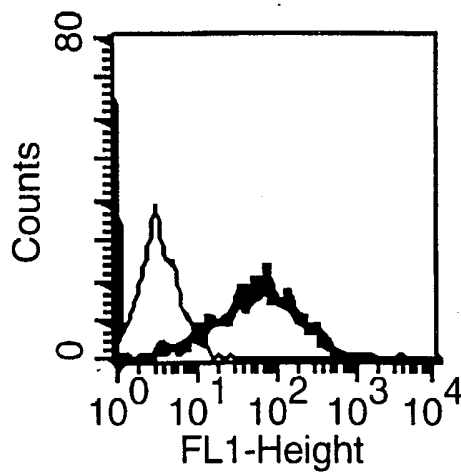
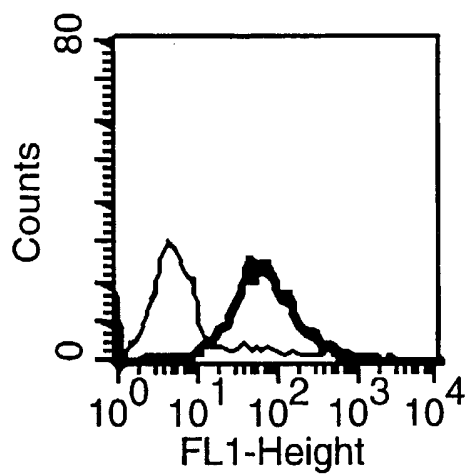


Figure 49 a

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**FRACTION 20**

3T6/CD32/B7.1



3T6/CD32/B7.2

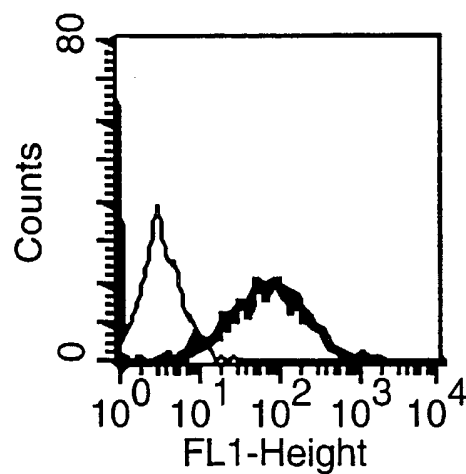
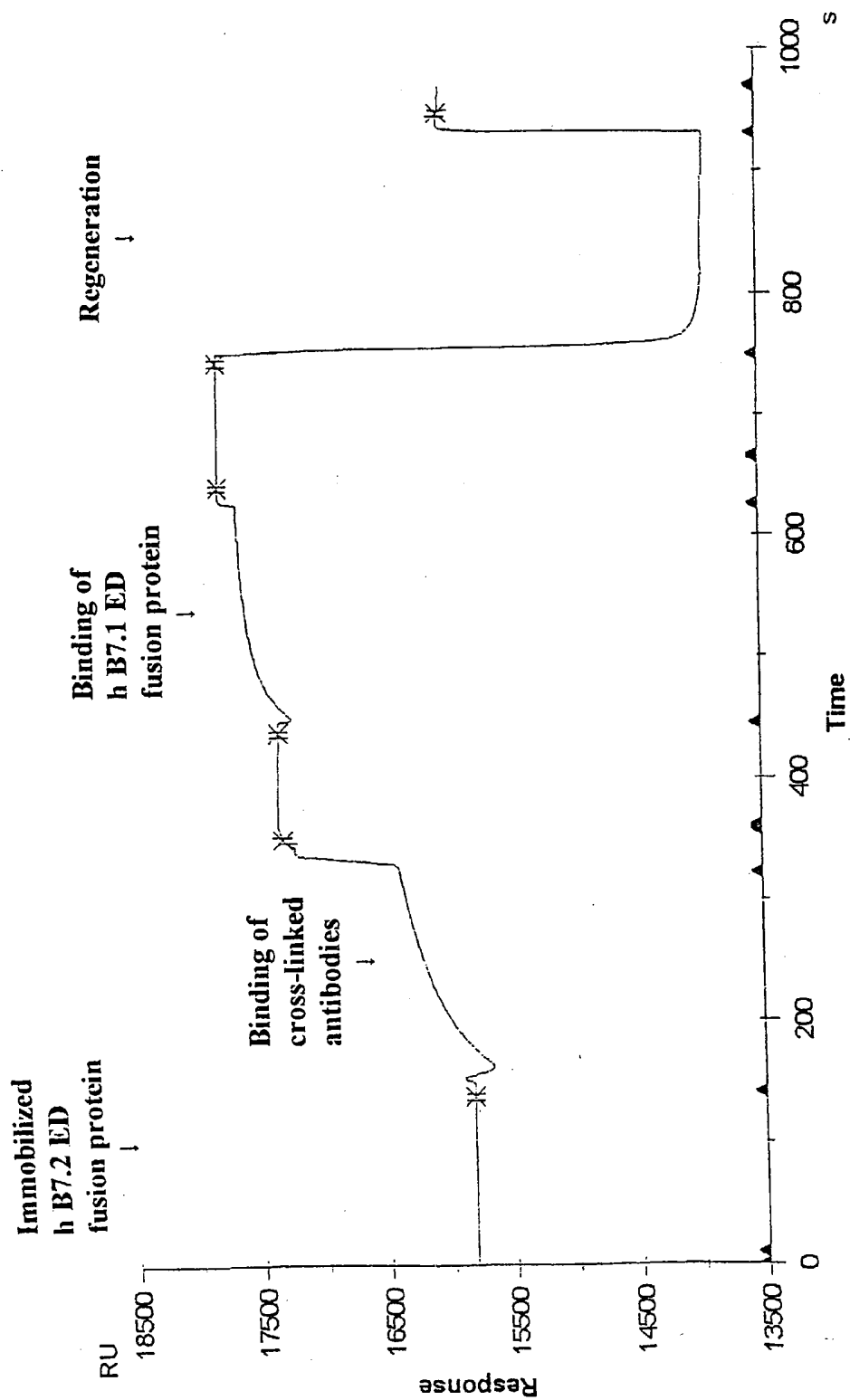


Figure 49 b

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Figure 50 - cont'd

Fc	Time	Window	AbsResp	SD	Slope	Baseline	RelResp	Id
4	138,5	5,0	15822,9	0,12	0,01	Yes	0,0	baseline (hB7.2 ED fusion prot.
4	351,5	5,0	17309,5	15,59	8,01	No	1486,6	crosslinked antibodies
4	438,5	5,0	17345,7	0,21	-0,09	No	1522,8	dissociation
4	641,5	5,0	17809,4	0,70	0,31	No	1986,5	hB7.1 ED fusion protein
4	744,5	5,0	17810,5	0,14	-0,05	No	1987,6	dissociation
4	948,5	5,0	16041,4	3,66	-1,92	No	218,5	regeneration (glycine pH 2.0)